

Hypoglycemic Brain Damage

An Experimental Neuropathologic Study in the Rat

Roland N. Auer



Lund 1985

From the Laboratory of Experimental Brain Research,
University of Lund, Sweden

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...Imagination bodies forth
The forms of things unknown.

William Shakespeare, A Midsummer Night's Dream

Doctoral thesis
Printed in Sweden
Studentlitteratur
Lund 1985

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Dedicated to my mother

This thesis is based on the following papers, which will be referred to by their Roman numerals in the text:

- I. Auer RN, Olsson Y, and Siesjö BK: Hypoglycemic Brain Injury in the Rat. Correlation of Density of Brain Damage with the EEG Isoelectric Time: A Quantitative Study. *Diabetes* 33:1090-1098, 1984.....41
- II. Auer RN, Wieloch T, Olsson Y, and Siesjö BK: The Distribution of Hypoglycemic Brain Damage. *Acta Neuropathol.(Berl.)* 64:177-191, 1984.....51
- III. Auer RN, Kalimo H, Olsson Y, Siesjö BK: The Temporal Evolution of Hypoglycemic Brain Damage. I. Light and Electron Microscopic Findings in the Rat Cerebral Cortex. *Acta Neuropathol.(Berl.)* (in press).....67
- IV. Auer, RN, Kalimo H, Olsson Y, Siesjö BK: The Temporal Evolution of Hypoglycemic Brain Damage. II. Light and Electron Microscopic Findings in the Hippocampal Gyrus of the Rat. *Acta Neuropathol.(Berl.)* (in press).....101
- V. Kalimo H, Auer RN, Siesjö BK: The Temporal Evolution of Hypoglycemic Brain Damage. III. Light and Electron Microscopic Findings in the Rat Caudoputamen. *Acta Neuropathol.(Berl.)* (in press).....133
- VI. Auer RN, Hall P, Ingvar M, Siesjö BK: Hypotension as a Complication of Hypoglycemia Leads to Enhanced Energy Failure But No Increase in Neuronal Necrosis. (submitted to *Stroke*).....169
- VII. Auer RN, Kalimo H, Olsson Y, Wieloch T: The Dentate Gyrus in Hypoglycemia. Pathology Implicating Excitotoxin Mediated Neuronal Necrosis. (submitted to *Acta Neuropathologica*).....193



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INTRODUCTION

Glucose is the predominant fuel consumed by the brain under normal circumstances. Delivery of both oxygen and glucose via the cerebral circulation is a constant requirement for unperturbed cerebral metabolism and function. Quite naturally, it has been tacitly assumed that both ischemia and hypoglycemia are therefore cerebral insults of a similar or identical nature, killing neurons by depriving them of either oxygen or glucose and thereby producing structurally equivalent results in the brain. This tenet is challenged in the present thesis.

Biochemical results which have emerged in recent years have already delineated significant differences between the neurochemical pathology of ischemia and hypoglycemia. However, the present thesis examines the anatomical pathology resulting from severe hypoglycemia, delineating it as distinct from existing descriptions of ischemic brain damage. Quantitative and qualitative approaches are used, with an emphasis on clues to mechanisms of neuronal necrosis.

BACKGROUND

The discovery of insulin sixty years ago generated the initial question of whether hypoglycemia could injure the brain, although the problem was then phrased in terms of the effects of insulin rather than of hypoglycemia. Insulin overdose in humans treated for diabetes (1) led to keen interest in the effects of insulin on the brain. Interest was further augmented by the introduction of "insulin shock" therapy by Sakel in 1934 (2). Insulin was given to humans for schizophrenia (2) as well as for acne, morphinism, and other conditions (3). Since such therapy led to behavioural changes and sometimes death of the patient (4,5), a vigorous debate about the appropriateness of such therapy resulted, providing the impetus for research into the possible existence of hypoglycemic brain damage. Researchers thus studied the effects of insulin in rabbits

(6-8), dogs (9,10), and monkeys (11) while pathologists reported autopsy findings from clinical cases of severe hypoglycemia in humans (4,5,12,13).

Most of the experimental pathological studies of the brain in hypoglycemia were thus performed in the 1930's. These early studies were physiologically uncontrolled. Insulin was used in all of them, but blood pressure, respiration, or, remarkably, blood glucose levels, were not monitored. Insulin itself was felt to be toxic. Attempts to prove this included insulin injections into the brain and ventricles (10). Insulin was even injected into the brain post mortem (10).

Whatever their shortcomings, these early studies had shown unequivocal evidence of neuronal loss in both animals (6-11) and humans with hypoglycemia (4,5,12-14), and the practical and pressing question of whether "insulin shock" therapy could permanently damage the brain appeared to have been answered in the affirmative. In the present context, a prescient observation must be mentioned, although it was later forgotten or ignored. Two research groups noticed a relation of hippocampal damage to the ventricle, either neuronal necrosis (8), or gliosis (11), but only the American neuropathologist Arthur Weil and his collaborators offered in 1938 the obvious explanation of a toxic substance carried in the fluid of the brain (8). This observation is noteworthy here, in view of the pathologic findings of the present thesis, which suggest that a tissue and cerebrospinal fluid borne excitotoxin, produced mainly in the telencephalic cortex, mediates hypoglycemic neuronal necrosis.

Research in this area subsequently lay relatively dormant. Hypoglycemic brain damage was subsequently considered to be similar or identical to that seen in ischemia (7,15-18), and it was assumed that neurons could be killed by depriving them of either oxygen or glucose, with similar effects on the brain.

Not until the 1970's did anatomical research in hypoglycemia begin again in full swing. Insulin was administered to monkeys in two pathological studies and neuronal loss was demonstrated in the brain (19,20). Biochemical studies were undertaken (21-27), and suggested important differences between ischemia and hypoglycemia. These include for example, the state of oxidation-reduction of tissue redox systems and the degree of energy failure. Cellular redox systems are reduced in ischemia due to lack of oxygen, but are oxidised in hypoglycemia (23-25). Energy failure is present in hypoglycemia (22,24,28) but is less severe than in ischemia due to oxidation of endogenous substrates derived from the brain itself (21,23-25). Also, the acidosis of ischemia contrasts with the alkalosis of hypoglycemia. Alkalosis during hypoglycemia (29,30) is due to the incapacity of the tissue to form lactate, due to a lack of glucose substrate, and to the production of ammonia from amino acid catabolism (21,24,25,27). Tissue infarction, with destruction of astrocytes and endothelial cells, appears related to lactic acid accumulation (31,32). Since acidosis is not a feature of hypoglycemia, it is noteworthy that infarction was not seen in any animals described in this thesis, thus indirectly corroborating the evidence linking lactic acidosis and infarction.

The most recent pathological results in hypoglycemia described dark neurons (28,33), and thus proved controversial (34,35). Dark neurons, assumed by several authors to be always due only to poor fixation (18,36), were described during and shortly after hypoglycemia (28,33). However, the tissue in these experiments was fixed by perfusion, and the microcirculation was free of red blood cells, suggesting the problem did not lie in inadequate fixation. Still, the fate of these dark neurons could not be ascertained in these experiments, due to the short recovery times used. Their significance as necrotic neurons destined for removal from the tissue, or alternatively as neurons with the potential for recovery, could not be determined.

OBJECTIVES

It was the purpose of this set of experiments to develop a model of insulin induced hypoglycemia in the rat, which had firstly adequate physiologic monitoring during the hypoglycemic insult, and secondly the possibility of long term survival. Without adequate physiologic monitoring, the perennial problem of being able to ascribe the consequent brain damage to complicating factors such as hypoxia or epilepsy rather than hypoglycemia, would cloud all interpretations of the results. Without long term survival, it could not be stated with certainty whether given pathologic changes would result in neuronal necrosis and disappearance of the neuron from the tissue.

The second aim was a comprehensive analysis of the quantitative and qualitative pathologic data generated, with regard to mechanisms of neuronal necrosis. Although not a primary goal of the present thesis, a comparison of the pathologic data presented here for hypoglycemia with the existing data on the pathology of ischemia, will reveal to the careful reader marked differences between these two forms of brain damage.

METHODS

Two basic paradigms were used. In one, factors changing the quantity of brain damage were manipulated, and neuronal cell counts were used in assessing the degree of consequent brain damage. This paradigm is used in papers I and VI. The rationale of this approach stems from the postulate that manipulation of a parameter important in causing neuronal necrosis, should be reflected in either an increase or a decrease in quantitated neuronal necrosis.

In the second paradigm, the insult was kept as constant as possible from animal to animal, and the quality of pathological changes was assessed as a function of time, using both light and electron microscopy. This paradigm was used in papers II, III, IV, V, and VII. It is the intent with this approach to overcome a basic

weakness in all methods of anatomical pathology: That any given change represents but an instant in time, the significance of which is unclear from the sole examination of the given tissue in one animal. The temporal evolution of cell changes was thus studied at tightly spaced time intervals to reveal the dynamics of cellular and tissue processes captured in only one instant in time.

A detailed description of the model can be found in paper I. Briefly, insulin (2 to 20 I.U./kg body wt) was given intraperitoneally to rats one hour before the experiment. Operation then consisted of intubation, ventilation with 1.0% halothane in a 70:30 N₂O:O₂ mixture, paralysis with suxamethonium, cannulation of the tail artery and veins, insertion of a central venous line, and placement of interhemispheric bipolar electroencephalogram (EEG) electrodes. The halothane was then discontinued, and blood gases and pH, blood pressure, blood glucose levels, and the EEG were subsequently monitored. After the desired period of EEG isoelectricity, the rats were given concentrated i.v. glucose over one minute. They were extubated after one to three hours, and were thereafter housed in animal cages with free access to tap water, until the time of sacrifice.

Perfusion fixation with phosphate-buffered intracardiac aldehydes was carried out after a heparin and saline rinse of the cerebral circulation, either before, after, or without spinal cord clamping. Such clamping had no effect on the results obtained. Since an immediate and substantial amount of blood returned immediately from the severed right atrium, and since the microcirculation was seen to be universally empty of red blood cells in the sections, perfusion fixation was deemed to be immediate and satisfactory.

For light microscopy (papers I, II, VI, VII), tissue was fixed with 4% formaldehyde, dehydrated, embedded in paraffin, sectioned at 8 μ m, and stained with acid fuchsin/cresyl violet. For electron microscopy, and for light microscopy in soft plastic embedding



medium (papers III, IV, V, VII), tissue was fixed with 3% glutaraldehyde, dehydrated, and embedded in either methacrylate polymer (light microscopy) or epoxy resin (electron microscopy).

Quantitative results were produced by direct visual counting of acidophilic neurons in the cerebral cortex, hippocampus, and caudate nucleus of subserially sectioned paraffin-embedded brains. Sectioning intervals were approximately 100 to 200 μ m, making it possible to consistently obtain identical portions of these regions for quantification. Since the entire rat brain was embedded, sampling problems inherent when only portions of the brain are examined were obviated.

Qualitative results were produced by analysis of sections on conventional light and electron microscopes. Light micrographs were produced on a Leitz Orthoplan photomicroscope with a zoom lens attachment, and electron micrographs on a Japanese Electron Optics Limited JEM 100C electron microscope.

RESULTS AND DISCUSSION

Paper I

In paper I, it was found that rats were able to indefinitely survive up to 60 minutes of cerebral isoelectricity due to hypoglycemia. No attempts were made to examine even longer periods with a flat EEG, since rats were already sick (see below) after this dose of insult. These results accord well with previous observations, since 30 minutes of "insulin coma" was the desired therapeutic interval for the insulin shock therapy popular in the 1930's.

Although cerebrally sluggish and peripherally flaccid, the awake rats did demonstrate normal behaviour, with a noteworthy total absence of seizures in over 150 rats. When rats died, usually after 60 min isoelectricity, they did so 8 to 24 hrs after the experiment, of flaccid respiratory paresis and eventual apnea. This result is noteworthy in view of the fact that anterior horn cells

were the commonest neuronal type in the spinal cord to undergo necrosis (papers I, II). Clinical reports in man have also noted a delayed onset of lower motor neuron weakness due to hypoglycemia (37-39).

It was also discovered that EEG isoelectricity was a necessary prerequisite for neuronal necrosis to occur. Furthermore, the number of necrotic neurons increased in rough proportion to the duration of EEG isoelectricity. There was considerable overlap between groups, however. For example, some rats showed more necrotic neurons after 20 minutes than others after 40 minutes with a flat EEG. The blood glucose levels during isoelectricity ranged from 1.5 mM down to one tenth this value, and showed no relation to the consequent brain damage (see table 3, paper I). Likewise, the dose of insulin was found to be irrelevant to the resulting brain damage.

The absolute blood glucose level and the dose of insulin used to produce it are thus not related to the onset of hypoglycemic brain damage. The level of hypoglycemia is important only to the extent that it must be under roughly 1.5 mM for the onset of EEG isoelectricity, the critical event heralding neuronal necrosis, to occur.

Paper II

In paper II the distribution of hypoglycemic brain damage was examined after one week survival. Necrotic neurons were found to have a dissimilar distribution to that seen in ischemia, being related to the subarachnoid, cisternal and ventricular spaces of the CSF, leading to the hypothesis of a fluid-borne toxin.

In the cerebral cortex, this pattern was manifest as a superficial cortical distribution of neuronal necrosis, becoming especially clear after insults of 40 to 60 minutes isoelectricity.

Due to the lack of a pia-CSF barrier, there is free reflux of CSF into the superficial cerebral cortex from the subarachnoid space (40).

In the hippocampus, the first structure consistently affected was the dentate gyrus, already after 20 minutes. Subicular, and then CA1 damage progressed from medial to lateral with worsening insults. The dentate crest and external blade showed necrosis first, in a pattern resembling published accounts of labelling of the dentate gyrus produced by subarachnoid injections of tracer (41).

In the caudate nucleus, damage was worst laterally if mild, but superiorly if damage was severe. Thus, different patterns were seen, depending on the severity of the insult, even in opposite hemispheres of the same animals when markedly asymmetric damage was present. The subependymal zone of the caudate, where fascicles of white matter are absent, showed no neuronal necrosis, even if damage was most severe. This, and the changing locus of the worst affected region within the caudate in relation to the total amount of damage, argues against a neuroanatomical basis for the distribution. A toxic substance carried to the caudate in the white matter tissue fluid seems the most coherent hypothesis, explaining thereby also the delayed onset of caudate damage (see below), following that in the cerebral cortex and hippocampus.

The amygdaloid nuclei showed neuronal necrosis in their portions near the ventricles, and the cerebellum showed necrosis of Purkinje cells near the foramen of Luschka, implying the presence of a neurotoxin in the ventricular system as well. The cerebellum shows better preservation of the energy state during hypoglycemia and suffers a less severe metabolic insult (27) than the cerebrum, due to very efficient transport of glucose from the blood into the cerebellum (42).

Paper III

The fate of previously reported dark neurons was examined in the cerebral cortex in paper III. The criteria of absent red blood cells in the microcirculation was taken as evidence of adequate fixation.

Dark neurons were present acutely, as previously reported, in all layers of the cerebral cortex. Some of these, and also some neurons of normal electron density, contained swollen mitochondria. The dark neurons had the typical appearance usually considered to be due only to poor fixation, consisting of hyperchromatic cytoplasm and corkscrew-like processes. Special darkroom procedures, resulting in an intentionally lighter rendition of the perikaryon, revealed preservation of the structure of subcellular organelles. This suggested recovery was possible in spite of the very dark appearance with the acidic and basic stains used for light microscopy, and the metal stains for electron microscopy. The non-specific hyperchromasia of the dark neurons to all types of stains used suggests a concentration of cytoplasmic constituents rather than a specific chemical alteration of the cell increasing the affinity for dye.

The evolution of these dark cells proved predictable, and they demonstrated recovery in the deeper layers, but necrosis in the superficial layers, giving rise to the final pattern of cellular damage seen at one week survival in paper II. Volumetric recovery of neuronal perikarya was seen in the deeper cortical layers, in spite of early mitochondrial swelling and dark cell transformation.

Dark neurons were thus considered to represent reversible cellular pathology, not due to inadequate fixation, as the microcirculation was empty of red blood cells. Dark neurons appeared in the cerebral cortex shortly after the onset of isoelectricity, known to be a harbinger of cell damage (paper I). In the light of the hypothesis that hypoglycemic neuronal necrosis

is mediated by an excitotoxin, it is noteworthy that dark neurons have been shown to be the acute neuropathology seen after excitotoxin administration (43,44).

Superficially, in contrast, a pronounced affinity for acid dyes developed in neurons by 6 to 8 hours under the light microscope, corresponding to the time of appearance of mitochondrial flocculent densities and cell membrane breaks under the electron microscope. These cells were consistently removed from the tissue after one to several weeks, and hence were considered necrotic. Since mitochondrial flocculent densities have been shown to represent denatured protein (45), and protein is the main cellular constituent stained by acid dyes, the most likely explanation for the simultaneous appearance of both mitochondrial flocculent densities and cellular acidophilia is some alteration in cellular proteins associated with cell death.

Paper IV

Here, the pyramidal cell band of the hippocampus was examined, including the subiculum, CA1 and CA3 zones. The reversibility of the mitochondrial swelling seen in the cortex could with certainty be deemed reversible in CA3 pyramidal neurons, as these cells never underwent necrosis after 30 min hypoglycemia. Such swelling was rampant in perikaryal mitochondria, in spite of subsequent survival of all these cells.

It was also possible in paper IV to compare the time of occurrence of neuronal death, ultrastructurally defined as large cell membrane breaks and mitochondrial flocculent densities, in the subicular and CA1 zones of the hippocampus in the same animal. Where damage was most severe, medially in CA1 and in the subiculum, neuronal death occurred by 2 hours. Laterally in CA1, however, neuronal necrosis seemed to be a lingering process, with preserved cellular structures seen at 3 weeks. However, the appearance was not identical to delayed neuronal death in ischemia (46-48). In

contrast to the stacked, abundant rough endoplasmic reticulum seen in delayed neuronal death in ischemia (47,48), the laterally placed CA1 pyramidal cells showed homogeneous nucleoplasm and only comparatively minor changes in the cytoplasm. Also, ischemic delayed neuronal death involves lysis of the cell by four days, whereas the lateral CA1 cells in hypoglycemia were still relatively intact at three weeks. The two forms of neuronal necrosis do not therefore seem directly comparable, further underscoring the differences between the pathology of ischemia and hypoglycemia.

Astrocytic and endothelial pathology was also studied, and changes were identical to those in the cerebral cortex, and hence are reported here. Both these non-neuronal cell types were preserved, although reactive changes were demonstrated. Necrosis of astrocytes or endothelial cells was never seen, even in animals showing the most severe damage. Necrosis of all non-neuronal as well as neuronal elements of the tissue constitutes infarction, and has been linked to lactic acidosis in the tissue (32). As explained previously in the background section, alkalosis, rather than acidosis, is present in hypoglycemia. Thus, if acidosis is responsible for infarction, then infarction should not be seen in hypoglycemia. This was universally the case in the present work.

Activation of astrocytes, however, was seen, comprising an increase in mitochondria after 12 hours. After days to weeks, the effete mitochondria formed residual bodies in the cytoplasm, and glial fibers became abundant. These changes suggest metabolic activation of astrocytes, with an initial increase in the turnover of mitochondria, and then subsequent degradation.

Endothelial cells were also altered. Numerous pits and vesicles were seen after several hours, along with endothelial microvilli. The significance of these changes is unclear.

Paper V

Here the changes in the caudate nucleus were examined through time. The caudate nucleus contrasted with the cerebral cortex and hippocampus in that dark neurons were absent during hypoglycemia. No significant pathology was seen during and shortly after 30 or 60 minutes of isoelectricity, at a time when dark neurons were ubiquitous in the cerebral cortex and hippocampus. The appearance of dark neurons in the caudate nucleus thus followed their appearance in other brain regions.

When dark neurons did appear, they predominated first laterally, and then in the more medial portion of the caudate. They became common by 3 to 4 hrs recovery and then, as in the cerebral cortex, transformed into acidophilic neurons. This transformation occurred by 6 to 8 hrs, and was accompanied by the ultrastructural features of cell death, consisting of cell membrane breaks and mitochondrial flocculent densities.

The caudate nucleus thus distinguished itself from the cerebral cortex and hippocampus in being the only area of the three showing no pathology for up to one hour of isoelectricity, or 30 minutes isoelectricity plus up to 30 minutes recovery. This was not due to lack of sensitivity of the caudate nucleus to the evolving insult: Subsequent neuronal necrosis developed consistently, and was dense.

The marked temporal delay in the appearance of dark, and then acidophilic neurons in the caudate as compared to other regions, combined with their appearance first in lateral locations, as well as the relationship of the caudate damage to the presence of white matter bundles in the caudate (see paper II), suggests the hypothetical toxin is not endogenously produced within the caudate, but rather is carried to the nucleus in the fluid transported by the white matter.

Paper VI

Since hypoglycemic brain damage was shown to have a distinct distribution from that of ischemia (49), it was possible to add these two insults to each other and distinguish the neuropathologic pattern as either hypoglycemic or ischemic. This was performed by lowering the blood pressure during the isoelectric period to levels (60 to 80 mm Hg) that result in focally ischemic blood flows, due to the loss of autoregulation during hypoglycemic isoelectricity. An area previously demonstrated to have ischemic flow rates during hypotension complicating hypoglycemia, due to a profound loss of vascular autoregulation, is the cingulate cortex (50). Conversely, an area shown to have relative preservation of cerebral blood flow even during moderate hypotension accompanying hypoglycemia was the septal nucleus (50).

Theoretically, either a hypoglycemic pattern, an ischemic pattern, or some combination of the two patterns might have been expected. The results, however, showed a pure hypoglycemic pattern of neuronal necrosis involving, for example, the crest of the dentate gyrus. Furthermore, areas showing the greatest loss of autoregulation, and therefore the greatest ischemia during the hypotension (eg. cingulate cortex), showed no increase in neuronal necrosis, and areas showing good blood flow during hypotension showed neuronal necrosis nevertheless. Also, total brain damage was not increased by the addition of hypotension to hypoglycemia, again underscoring the distinct and non-additive nature of the two insults. Production of the hypothetical toxin did not seem to be enhanced by hypotension.

Paper VII

Here the dentate gyrus was examined in detail, and changes in the dendritic trees were compared with those occurring simultaneously in the cell bodies and axons. Electron microscopy demonstrated the characteristic axon-sparing, dendritic lesion of excitotoxins (43).

In the neuropil of the stratum moleculare, dendrites were markedly swollen and contained swollen mitochondria at ten minutes of isoelectricity, a time at which the cell bodies were normal.

Subsequently, at thirty minutes isoelectricity, the perikarya showed abnormalities, with swollen mitochondria and endoplasmic reticulum. Thirty minutes after this, irrespective of whether glucose had been given, cell membrane irregularities had developed, and these subsequently evolved into frank membrane rupture by one hour of recovery. Mitochondrial flocculent densities appeared, and the neuron was removed by either cytolysis or macrophages in the subsequent weeks. Abnormalities in the axons of the dentate neurons, consisting of Wallerian degeneration, both in the hilus of the dentate and in the mossy fiber boutons in the CA3 zone, appeared after 12 hrs, long after dendritic and perikaryal changes had taken place.

It was thus possible to construct a sequence of cytopathologic changes resulting in neuronal necrosis at the dentate crest. This was possible due to the consistent necrosis at this location, in combination with the simple laminar arrangement of cell bodies and dendrites. The sequence revealed firstly dendritic abnormalities, then perikaryal abnormalities, and finally cytorrhesis and axonal Wallerian degeneration. The sparing of axons, with affectation of only dendrites in the neuropil, is characteristic of excitotoxins (43), due to their binding to post-synaptic dendritic receptors.

Together, the above results led to a reformulation and extension of the original hypothesis of Weil and his collaborators (8), that toxic substances dissolved in the fluids of the brain should be considered. It seems likely that when brain electrical activity ceases due to hypoglycemia, an endogenous excitotoxin is produced, chiefly by the neocortex and hippocampus, resulting first in dark neurons, already during isoelectricity, and then acidophilic neurons. Tissue fluid carrying the toxin into the caudate nucleus causes a dark to acidophilic neuron sequence later in that region.

Dendritic trees exposed to the toxin-bearing fluid undergo severe damage, which spreads toward the cell body, destroys cell membranes, culminating in neuronal necrosis. The resulting pattern of neuronal necrosis thus shows a relationship to the white matter and CSF pathways of the brain.

CONCLUSIONS

1. Acidophilic neuronal necrosis is absent in the brain in hypoglycemia unless a flat EEG has appeared, regardless of blood glucose levels.
2. Acidophilic neuronal necrosis increases in rough proportion to the duration of a flat EEG.
3. A duration of up to one hour without electrical brain function is survivable indefinitely in the rat.
4. The distribution of necrotic neurons consequent to hypoglycemia shows specific relationships to the white matter, and to the CSF spaces in the rat brain.
5. Dark neurons, and neurons with mitochondrial swelling, represent, in aldehyde-fixed tissue, acute, potentially reversible neuropathology.
6. Dark neurons, with hyperchromasia to all dyes, can evolve into acidophilic neurons, demonstrating a pronounced affinity especially for acid dyes.
7. Acidophilic neurons show ultrastructural features of cell death, consisting of mitochondrial flocculent densities and ruptured cell membranes, these features constituting the cellular "point of no return".
8. Neuronal death occurs more rapidly in areas where there is denser neuronal necrosis.
9. The toxic substance suggested by the light microscopy is an excitotoxin.

ACKNOWLEDGEMENTS

Professor Bo K. Siesjö has been my supervisor for the past 3 years. I am indebted to him for asking the right questions, and for allowing me to work on the answers using the pathologic techniques of my trade in his laboratory. His guidance in scientific writing was indispensable. Dr. Martin Ingvar gave me valuable scientific advice and invaluable assistance in development of the model, and introduced me to the computer. Vigorous discussions of a scientific nature with Dr. Tadeusz Wieloch will not be forgotten. It was a privilege to work with two neuropathological colleagues possessing great expertise, Dr. Hannu Kalimo and Dr. Yngve Olsson, both of whom accepted me and helped me as a fledgling researcher in their field. Without the extreme generosity of Yngve Olsson with his laboratory and staff, large portions of the light microscopy and all of the electron microscopy would have been impossible. I am grateful as well to Dr. Arne Brun for allowing me limitless use of his photomicroscope. His help and that of his technicians, Kerstin Sturesson and Elsie Andersson, was invaluable in developing the histologic methodology.

Without Heléne Wilhelmsson performing the experiments and Lille-Mor Lindeström, Marianne Forssén, and Maud Salomonsson cutting and staining the sections, few brains indeed would have been examined. Erik Arro removed bugs from the electron microscope, sometimes daily. I am thankful to Birgit Olsson for suggesting the beautiful acid fuchsin staining, for persisting with my soft plastic blocks until they were cut, and for creating such fine illustrations. The staff of the photography department of Lunds Lasarettet produced high quality retouched light photomicrographs, and Frank Bittkowski produced unbelievably crisp electron micrographs for publication. Drs. Hans Hedlund, Martin Ingvar, Arne Brun, Marika Kiessling, Peter and Margitta Pietz, Tadeusz Wieloch, Lucyna Schalén, Marie de France Wetterling, and Tohru Koide translated the summary.

Yvonne Hagberg and Gunvor Jacobsson smoothly obtained accommodation for me in Lund and Uppsala respectively, and Dr. Hannu Kalimo accommodated me in his home in Finland, also providing excellent tennis. My travels within the Nordic countries were thus always a pleasure.

The numerous friends I made here in Sweden cannot be mentioned, but they provided respite from work with sport and social activities. I am indebted to Dr. Rolando Del Maestro for initially getting me interested in research and connecting me with Sweden. I am thankful to my future bride, Iwona, for waiting so patiently in Poland between my visits, and to my mother and father in Canada, for waiting even longer for me to return. For the Fellowship from the Medical Research Council of Canada, which gave me unfettered support during my entire stay here in Sweden, I am grateful to the people of that organization, and to the people of Canada.

Summary

The anatomical pathology of hypoglycemic brain damage in the rat is described in a model allowing both tight control of physiological variables, as well as long term recovery and indefinite survival. No neuronal necrosis was seen in the brain unless the electroencephalogram went flat, regardless of the blood sugar level at which this occurred. Necrotic neurons increased in number in rough proportion to the duration of electrocerebral silence, but their number was not increased by lowering the blood pressure during or after hypoglycemia.

The distribution of necrotic neurons in the cerebral cortex, hippocampus, caudate nucleus and cerebellum was distinct from that previously reported in ischemia. Necrotic neurons showed a relationship to the white matter and cerebrospinal fluid pathways of the brain, suggesting the presence of a fluid-borne toxin in causing the neuronal necrosis.

Necrotic neurons in all brain regions were found to have a pronounced affinity for acid dyes by light microscopy. Such neurons showed the ultrastructural features of cell necrosis, consisting of mitochondrial flocculent densities and cell membrane breaks. The consistent removal of acidophilic neurons from the tissue after days to weeks further established these cells as necrotic.

Dark neurons were seen in the cerebral cortex and hippocampus, but not in the caudate nucleus, during hypoglycemia. Dark neurons appeared in the recovery period in the caudate. Dark neurons developed into acidophilic neuronal necrosis in all locations except the deeper layers of the cerebral cortex, where these cells by were found to be capable of recovering completely.

In the dentate gyrus, swollen dendrites with swollen mitochondria were seen in the stratum moleculare. Intermediate axons were spared. Dendritic abnormalities were seen early in the course of the cellular lesion, prior to the appearance of cell membrane and cytoplasmic abnormalities in the perikaryon. Although dendrites were severely damaged, intermediate axons were preserved. Such changes regularly evolved into neuronal necrosis at the dentate crest, a portion of the gyrus exposed to the subarachnoid cistern above the third ventricle. The axon-sparing, dendro-perikaryal nature of the lesion indicates the neurotoxin, suggested by the light microscopy, is an excitotoxin, mediating neuronal necrosis in hypoglycemia.

Sammanfattning på svenska

Den patologiska anatomin vid hypoglykemisk hjärnskada hos råttor beskrivs för en modell, som tillåter kontroll av fysiologiska variabler och dessutom möjliggör långtidsöverlevnad.

Neuronskada företåg ej om inte EEG blev isoelektriskt, oavsett vid vilken blodsockernivå detta inträffade. Antalet nekrotiska neuron ökade i proportion till längden av den isoelektriska perioden, men ökning av antalet nekrotiska neuron noterades ej om blodtrycket sänktes under eller efter den hypoglykemiska perioden.

Distributionen av nekrotiska neuron i cortex, hippocampus, nucleus caudatus och cerebellum var skild från det mönster som tidigare finns beskrivet för ischemi. Nekrotiska neuron hade en specifik relation till den vita substansen och cerebrospinalvätskan, vilket gav en antydning om att ett vätskeburet toxin kunde vara orsaken till att neuronerna blev nekrotiska.

Nekrotiska neuron identifierade i ljusmikroskop, hade stor affinitet för sura färgämnen. Sådana neuron hade ultrastrukturella egenskaper, som kännetecknar cellnekros, i form av flocculära mitokondrier förtätningar och brott på cellmembranen. De acidofila neuronerna försvann ur vävnaden efter dagar till veckor, vilket bevisade att dessa neuron var nekrotiska.

Mörka neuron fanns i cortex och hippocampus men inte i nucleus caudatus under hypoglykemi-perioden. Dessa neuron blev acidofila efter flera timmar, utan de i de djupa cortexlagren där förändringen var nästan helt reversibel. I caudoputamen utvecklades mörka neuron efter den isoelektriska perioden. Dessa neuron blev också acidofila efter 6-8 timmar.

Ultrastrukturellt sågs i gyrus dentatus svullna mitokondrier och dendriter i molekyllagret. Mellanliggande axoner var opåverkade. Dendritabnormiteter sågs tidigt under utvecklingen av cellskadan, innan man såg tecken på skada i cellmembranen och cytoplasman. Sådana förändringar utvecklades regelmässigt till neuronal nekros i spetsen av gyrus dentatus, som ligger intill subarachnoidalrummet ovanför tredje ventrikeln. Det faktum, att axonerna var intakta i detta i övrigt skadade område talar för det neurotoxin, som de ljusmikroskopiska studierna antyder, och att detta är ett "exitotoxin", som utlöser neuronal nekros vid hypoglykemi.

Zusammenfassung auf deutsch

Die vorliegende Arbeit beschreibt die pathologische Anatomie des hypoglykämischen Hirnschadens bei der Ratte. Dazu wurde ein experimentelles Modell von Insulin-induzierter Hypoglykämie entwickelt, das erstens eine Durchführung der Versuche unter physiologisch kontrollierten Bedingungen erlaubt und zweitens eine Langzeitwiedererholung der Tiere ermöglicht. Ganglienzellnekrosen traten erst nach Einsetzen eines isoelektrischen EEG's auf, unabhängig vom jeweiligen Blutzuckerspiegel. Die Anzahl der irreversibel geschädigten Neurone nahm annähernd proportional zur Dauer der isoelektrischen Periode zu, wurde jedoch nicht durch ein Absinken des Blutdrucks während oder nach der Hypoglykämie beeinflusst.

Die regionale Verteilung der Ganglienzellnekrosen unterschied sich deutlich vom bisher beschriebenen Schädigungsmuster bei Ischämie. Nekrotische Neurone in der Großhirnrinde, im Hippocampus, im Nucleus caudatus und im Kleinhirn waren vorwiegend in enger Nachbarschaft zur weißen Substanz und zur Hirnoberfläche lokalisiert, insbesondere in den oberen Cortexschichten und der Spitze des Gyrus dentatus. Dieses Verteilungsmuster könnte ein Hinweis auf das Vorliegen einer neurotoxischen Substanz im Liquor cerebrospinalis sein.

Nekrotische Ganglienzellen in allen Hirnregionen zeichneten sich lichtmikroskopisch durch eine ausgeprägte Affinität zu sauren Farbstoffen, insbesondere Säurefuchsin, aus. Solche azidophilen Neurone zeigten ultrastrukturell alle Charakteristika der Zellnekrose, einschließlich flockiger Verdichtungen in den Mitochondrien und Defekten der Zellmembran. Das Auftreten einer Abraumreaktion mit Phagozytose nach einigen Tagen bis Wochen beweist, daß es sich bei azidophilen Neuronen um Nekrotische Zellen handelt.

Während der Hypoglykämie fanden sich sogenannte "dark neurons" in der Großhirnrinde und im Hippocampus, jedoch nicht im Nucleus caudatus. Mit Ausnahme der Zellen in den unteren Rindenschichten der Großhirnrinde, die eine Wiedererholung zeigten, entstanden aus diesen "dark neurons" überall azidophile nekrotische Zellen. Im Nucleus caudatus traten "dark neurons" zu einem späteren Zeitpunkt auf als in anderen Hirnregionen, entwickelten sich in dieser Lokalisation jedoch anschließend ausnahmslos eine Azidophilie.

Im Stratum moleculare des Gyrus dentatus fanden sich Dendriten und Mitochondrienschwellungen, dazwischen verlaufende Axone waren jedoch nicht geschädigt. Abnormalitäten in den Dendriten waren bereits zu einem frühen Zeitpunkt der Zellläsion sichtbar, d.h. vor dem Auftreten von Zytoplasma- und Zellmembranveränderungen im Perikaryon. Die beschriebenen Veränderungen gingen an der Spitze des Gyrus Dentatus - einer Region, die dem Subarachnoidalraum oberhalb des III Ventrikels benachbart ist - regelmäßig in Ganglienzellnekrosen über. Die Tatsache, daß Axone von der Schädigung ausgespart blieben, weist darauf hin, daß es sich bei dem Aufgrund der lichtmikroskopischen Befunde vermuteten Toxin um eine exzitotoxische Substanz handelt, die als Mediator bei der Entwicklung der hypoglykämischen Zellschädigung fungiert.

Streszczenie po polsku

W modelu, który pozwala zarówno na ścisłą kontrolę warunków fizjologicznych jak i na długoterminowe wyzdrowienie i przeżycie, studiowano patologię anatomiczną hipoglikemicznego uszkodzenia mózgu szczura. Niezaobserwowano nekrozy neuronów o ile elektroencefalogram nie był izoelektryczny, niezależnie od aktualnego poziomu glukozy we krwi. Liczba uszkodzonych neuronów wzrastała proporcjonalnie do czasu trwania nieaktywności mózgu, jednakże liczba ta nie zwiększała się po obniżeniu ciśnienia krwi w trakcie lub po okresie hipoglikemii.

Rozkład nekrozy komórek nerwowych w korze mózgowej, zakręcie hippokampa, jądrze ogoniastym i mózdziku różnił się zdecydowanie od zmian uprzednio opisanych w stanie niedotlenienia. Uszkodzone komórki nerwowe wykazywały powiązanie z białą substancją i drogami płynu mózgowo-rdzeniowego, co sugeruje że martwice neuronów została spowodowana toksynami przeniesionymi za pośrednictwem płynu mózgowo-rdzeniowego.

Uszkodzone neurony we wszystkich częściach mózgu wykazywały znaczne chłonięcia barwników kwasowych. Posiadały one takie cechy ultrastrukturalne wskazujące na nekrozę komórkową, t.j. złądzakowane zaciemnienia w mitochondriach i porożrywane błony komórkowe. Bezustanne usuwanie kwasochłonnych neuronów z tkanki mózgowej po kilku dniach-tygodniach dodatkowo utrwalało stan nekrozy tych komórek.

Ciemne neurony zaobserwowano w okresie hipoglikemii w korze mózgowej i w zakręcie hippokampa, lecz nie w obrębie jądra ogoniastego. Ciemne neurony pojawiły się w jądrze ogoniastym w okresie zdrowienia. Ciemne neurony uległy kwasochłonnej martwicy we wszystkich studiowanych obszarach z wyjątkiem głębokich warstw kory mózgowej, gdzie stwierdzono możliwość ich pełnego wyzdrowienia.

W opasce zębatej zakrętu hippokampa obrzmiałe dendryty i mitochondrie zaobserwowano we stratum moleculare. Axony pośrednie były dobrze zachowane. Anomalie dendrytów zaobserwowano we wczesnym stadium uszkodzenia komórkowego, przed pojawieniem się zmian błony komórkowej i cytoplazmy w perikarionie.

Axony pośrednie były dobrze zachowane mimo znacznego uszkodzenia dendrytów. Zmiany tego typu regularnie przechodziły w nekrozę komórkową w obrębie opaski zębatej, t.j. części zakrętu hippokampa eksponowanego na zbiornik podpajęczynówkowy ponad trzecią komorą.

Natura uszkodzenia dendro-perikarialnego z zachowaniem aksonów wskazuje na obecność neurotoksyny, będącej ekzitotoksyną, która pośredniczy w nekrozie neuronów w hipoglikemii.

Résumé en français

L'anatomie pathologique de la lésion cérébrale hypoglycémique chez le rat est décrite selon un modèle qui permet le contrôle de variables physiologiques et qui de plus rend possible le rétablissement et la survivance indéfinie.

La condition essentielle pour que les neurones soient endommagés était que l'électroencéphalogramme devienne isoélectrique à cause de l'hypoglycémie. Le niveau de sucre dans le sang tel quel n'ayant aucune influence sur l'endommagement des neurones. Le nombre de neurones nécrosés augmentait en proportion avec la durée de la période isoélectrique, tandis qu'aucune augmentation du nombre des neurones nécrosés n'était à noter si la pression sanguine diminuait pendant ou après la période hypoglycémique.

La distribution des neurones nécrosés dans le cortex, l'hippocampe, le noyau caudé et le cervelet se différenciait du modèle antérieurement décrit de l'ischémie. Les neurones nécrosés se situaient près de la substance blanche et du liquide cérébro-spinal, ce qui portait à croire qu'une toxine transportée par le liquide devait être à l'origine de la nécrose des neurones.

Les neurones nécrosés pouvaient être identifiés au microscope par leur grande affinité aux colorants acides. De tels neurones avaient sous le microscope électronique des caractéristiques qui identifient la nécrose cellulaire, telle des condensations de mitochondries et des brisures de la membrane cellulaire. Les neurones acidophiles disparaissaient des tissus après des jours et des semaines, ce qui prouve que ces neurones étaient nécrosés.

On trouvait des neurones noirs dans le cortex et l'hippocampe, mais pas dans le noyau caudé, durant la période hypoglycémique. Ces neurones devinrent acidophiles après plusieurs heures, cependant la transformation en neurones noirs dans les couches profondes du cortex était presque entièrement réversible. Dans le noyau caudé, se développèrent des neurones noirs après la période isoélectrique. Ces neurones devinrent aussi acidophiles après 6-8 heures.

Sous le microscope électronique il y avait dans le gyrus dentatus des mitochondries et des dendrites enflées dans la couche moléculaire. Les axones entre étaient intacts. Les abnormités des dendrites étaient visibles tôt durant le progrès de l'endommagement cellulaire, avant même qu'on ne voit de dommage à la membrane cellulaire et au cytoplasme qui entoure le noyau de la cellule. De telles transformations se développaient régulièrement en nécroses de neurones dans la pointe du gyrus dentatus, la partie qui est située près de l'espace subarachnoïdien au-dessus du troisième ventricule. Le fait que les axones étaient épargnés dans cet endroit sinon endommagé démontre que la neurotoxine dont les études microscopiques laissaient entendre l'existence est une "excitotoxine".

要約

低血糖の脳障害像を、ラットを用いて、解剖病理学的に検討した。尚、このラットモデルは生理学的要因を制御することができると共に、長期慢性実験も可能なものである。神経細胞の壊死は、血糖値が十分に低値であっても、脳液がフラクトにならない限り認められることはなく、かつ、この脳液時間とほぼ対応して増加した。又、低血糖時あるいは回復時に低血圧にしても壊死率は増えることはなかった。

大脳皮質、海馬、尾状核及び小脳での神経細胞の壊死は、脳虚血で報告されている成績とは明らかに異なって分布していることが判明した。すなわち、低血糖の細胞壊死は自質な並びに脳脊髄液の分布と相関性が認められたことから、脊髄液中に存在しているある種の"toxin"が原因物質であろうと考えられた。

脳の領野のいかにかわらず光学顕微鏡において、これら壊死におちいた神経細胞は、酸性色素に極めて親和性が高いことがわかった。又、そのような神経細胞の膜外構造として、ミトコンドリアでの密度の高い綿状物質あるいは細胞膜の崩壊などが特徴的であった。これら酸性親和性の神経細胞は、数日後、数週間後には組織から完全に脱落してしまうという事実からしても、壊死状態にあると考えられた。

低血糖時での、いわゆる"dark neuron"は、大脳皮質及び海馬では認められたものの、尾状核では認められることはなかった。しかしながら、低血糖から回復する時期には、尾状核でも出現した。大脳皮質を除く全ての神経細胞では、"dark neuron"はやがて酸性色素親和性の壊死神経細胞へと変化していった。大脳皮質深層部での神経細胞は、完全に回復するものであることがわかった。

歯状回においては、ミトコンドリアの膨化をもなった樹状突起の膨化が分子層で認められた。軸索では、このような変化はほとんど認められず、又、樹状突起でのこのような変性は、細胞障害の比較的初期段階に出現し、神経細胞体の細胞膜及び細胞質が変化するまでに認められた。このように樹状突起が極度に変性をうけ、軸索はほぼ正常であるという所見は、第3脳室上のフモ膜下腔槽に面した歯状回にのみ一致して認められた。すなわち、光学顕微鏡で得られたこれらの成績—軸索はほぼ正常であるにもかかわらず、樹状突起及び神経細胞体が障害を受けたという実験事実—は、低血糖による神経細胞の壊死は、"excitotoxin"により引き起こされるものであることを示唆している。

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Hypoglycemic Brain Injury in the Rat

Correlation of Density of Brain Damage with the EEG Isoelectric Time: A Quantitative Study

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SUMMARY

Thirty-eight male Wistar rats were exposed to insulin-induced hypoglycemia resulting in periods of cerebral isoelectricity ranging from 10 to 60 min. Plasma glucose levels during cerebral isoelectricity ranged from 0.12 mM to 1.36 mM. Control rats were injected with insulin, but hypoglycemia was terminated with glucose at the stage of large δ -wave EEG slowing. After recovery, the rats were allowed to wake up and survive for 1 wk.

The number of dying neurons was assessed with acid-fuchsin/cresyl-violet-stained, whole-brain, subserial sections using direct visual counting of acidophilic, cytoelastic neurons. Brains from control rats that were not allowed to become isoelectric showed no dying neurons. Ten minutes of cerebral isoelectricity produced very minimal brain damage. The density of neuronal necrosis was positively related to the number of minutes of cerebral isoelectricity up to the maximum examined period of 60 min, but showed no correlation with the blood sugar levels.

The cerebral cortex, hippocampus, caudate nucleus, spinal cord, and, to a lesser extent, cerebellar Purkinje cells were affected. The distribution of neuronal necrosis was not identical with that seen in ischemia, but, rather, suggested a CSF-borne neurotoxin operant in contributing to the pathogenesis of neuronal necrosis in hypoglycemic brain damage.

Neuronal death does not occur in hypoglycemia unless the EEG becomes isoelectric, whatever the blood sugar level. Serious brain damage does not occur until electrocerebral silence has been established for at least several minutes. Neuronal death accelerates after 30 min of EEG isoelectricity in the rat. Most animals

recovered well after up to 40 min of cerebral isoelectricity. DIABETES 1984; 33:1090-98.

Brain damage due to hypoglycemia acquires paramount importance in the treatment of diabetes, although hypoglycemia occurs in numerous, less common clinical conditions. Though the mechanism of neuronal death in hypoglycemia is as yet unclear, the fact that hypoglycemia may lead to permanent brain damage has been evident for a long time. Early workers studying the problem concentrated on the CNS effects of insulin,^{1,2} but found no simple relationship between the dose of insulin and the degree of brain damage incurred.

More recent workers have examined the degree and duration of hypoglycemia in relation to the clinical and neuropathologic consequences,³⁻⁵ as well as the relationship of the physiologic parameters to the neuropathologic sequelae,⁶ yielding a more satisfactory but still imperfect cause and effect relationship: both the degree and the duration of hypoglycemia exacerbated the clinical and neuropathologic consequences.

The critical event, however, heralding the onset of hypoglycemic brain damage has not been defined. The absence of brain damage in monkeys whose evoked potentials had not disappeared, in spite of prolonged hypoglycemia,³ suggests that the onset of electrocerebral silence may signal the onset of brain damage. Nevertheless, a study correlating the duration of cerebral isoelectricity and the severity of consequent brain damage in hypoglycemia has not been performed. Such information might be clinically useful, with regard to both prevention and prognostication of permanent brain damage.

This article describes: first, a new, physiologically controlled experimental model of hypoglycemia in the rat allowing for long-term recovery. Tight control of physiologic parameters was deemed essential to be able to attribute the consequent brain damage to hypoglycemia and not to ex-

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Received for publication 21 February 1984.

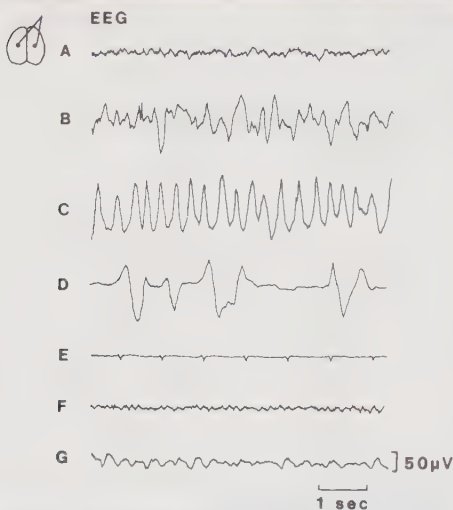


FIGURE 1. The EEG at various stages of hypoglycemia. (A) Normal. (B) One hour before isoelectricity, with slowing to the θ - δ range, and increased amplitude. (C) Rhythmic high-amplitude δ -slowing 20 min before isoelectricity. (D) Bursts of δ -activity alternating with total suppression of activity, heralding isoelectricity. (E) Isoelectricity with only artifacts visible. (F) Thirty minutes of recovery after 10 min of isoelectricity. No abnormalities are seen. (G) Sixty minutes of recovery after 60 min of isoelectricity. There is persistent slowing, but of lower amplitude than that seen before isoelectricity.

traneous insults such as hypoxia and hypotension. Extended recovery periods were deemed essential to allow for unequivocal neuropathologic features of neuronal death to develop. One week was the survival interval chosen after pilot studies.

Second, the period of cerebral isoelectricity will be related to the quantitated brain damage produced.

Last, a smoldering question in the field of hypoglycemic brain damage will be briefly addressed: is the distribution of hypoglycemic brain damage identical¹⁷ or not¹⁸ to that seen in anoxia-ischemia? A detailed histopathologic analysis of the intracerebral localization of hypoglycemic brain damage will be published elsewhere,⁹ but the distribution of CNS damage will be briefly presented here, with a correlation to clinical outcome.

MATERIALS AND METHODS

Experimental model. Male Wistar rats (SPF strain, Møllegaard's Avslaboratorium, Denmark) weighing between 310 and 495 g were allowed free access to tap water, food pellets (Astra-Ewos, Södertälje, Sweden), and digestive biscuits (Göteborgs Kex AB, Kungälv, Sweden) before the experiments. They were fasted overnight before the experiments, with access only to tap water.

Forty to sixty minutes before operation, the rats were given between 9 and 21 IU/kg of regular insulin (Actrapid, Novo

Industri A/S, Copenhagen, Denmark) intraperitoneally. They were immersed in 3% halothane (ISC Chemicals, Ltd., Avonmouth, United Kingdom) for 10 min, intubated (Intramedic PE 240 polyethylene tubing, Clay Adams, Parsippany, New Jersey), placed on a respirator (Braun, Melsungen, FRG), and ventilated with 0.8% halothane in a 2:1 N₂O:O₂ mixture throughout the operation.

Two tail veins and the tail artery were then cannulated with polyethylene tubing (Portex PE 50, Hythe, Kent, United Kingdom). A central venous catheter was inserted via the external jugular vein, to allow for rapid exsanguination and reinfusion of blood for blood pressure control. A continuous infusion of suxamethonium chloride (Celocurin, Vitrum AB, Stockholm, Sweden) in 50% Krebs-Henseleit solution was given through one vein by a syringe infusion pump (model 355, Sage Instruments, Cambridge, Massachusetts) at 2 mg/h, in a volume of 1 ml/h. The tail artery catheter was connected to a pressure transducer (Siemens-Elema AB, Solna, Sweden). To obviate bleeding problems, the animals were not heparinized, but the catheters were kept open by replacing the contents with heparin (100 IU/ml) after sampling.

Microelectrodes operating at 37°C were used to measure arterial PO₂, PCO₂ (Eschweiler and Co., Kiel, FRG), and pH (Radiometer, Copenhagen, Denmark) immediately after sampling. The rat was kept at a rectal temperature of $37.5 \pm 0.5^\circ\text{C}$ throughout the experiment by means of a 60-W light bulb at the required distance (usually 40–50 cm) from the semiprone animal. An interhemispheric, bipolar electroencephalogram (EEG) was recorded through two subcutaneous, bipolar needle electrodes. The blood pressure and EEG were continuously monitored on a multichannel recorder (Mingograph, Siemens-Elema AB, Solna, Sweden). After the above connections were made for monitoring physiologic parameters, the halothane was discontinued, the animal was ventilated on a 2:1 N₂O:O₂ mixture, and the period of cerebral isoelectricity was awaited.

Just before EEG isoelectricity, 0.1 mg of atropine (ACO Lakemedel AB, Solna, Sweden) was given intravenously. At

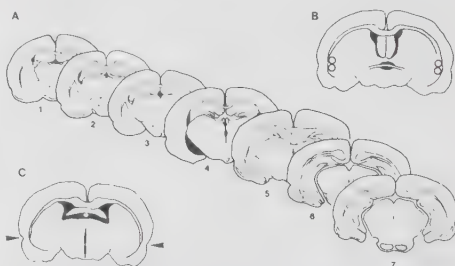


FIGURE 2. Coronal sections of brain demonstrating the standardized levels chosen from the subserial sections for neuronal counting. The hippocampus was sampled at eight levels throughout its septo-temporal extent (A), the most temporal level being on section 6. The caudate nuclei were quantified at the level of the septal nuclei at their widest point (B), with the location of counted fields indicated by circles. The cerebral cortex was counted at the level of the subornal organ (C), from the corpus callosum to the rhinal fissures (arrow-heads).

RELATION OF EEG ISOELECTRICITY TO HYPOGLYCEMIC BRAIN DAMAGE

the onset of cerebral isoelectricity, a timer was started. Two arterial blood samples were taken during the isoelectric period for fluorometric determinations of blood glucose levels using the hexokinase method.¹⁸ Blood glucose levels outside the isoelectric period were measured with Dextrostix reagent strips and a reflectometer system (Ames Co., Elkhart, Indiana).

Shortly after the onset of cerebral isoelectricity, a steady increase in the blood pressure occurred from the resting value of 90–110 mm Hg, which was held between 140 and 160 mm Hg by arterial bleeding. Blood was collected from the central venous catheter into a heparinized syringe, and was immediately reinfused via the second tail vein if the pressure fell. With this method, it was possible to maintain the blood pressure at 150 ± 10 mm Hg throughout the isoelectric period. Near the end of longer periods of isoelectricity, it was often necessary to reinfuse a major portion of the exsanguinate to keep the desired blood pressure of 150 mm Hg. The remaining blood was reinfused into the animal during the recovery period.

At the end of the desired period of EEG isoelectricity, recovery was induced with an infusion of 0.2 ml of 50% glucose, given by hand over 1 min. The suxamethonium drip was then substituted with 50% glucose at 1.5 ml/h. This infusion was tapered over the ensuing hours, and substituted with a 1:1 solution of 50% glucose and Krebs-Henseleit solution given at 0.5–1.0 ml/h overnight to maintain a plasma glucose between 5 and 10 mmol/L. Control animals were recovered in a similar fashion at the EEG stage of large δ -wave slowing or burst suppression (Figure 1).

Once the rat recovered spontaneous respiratory effort and made active, purposeful movements to remove the endotracheal tube, 100% oxygen was administered, the oropharynx was suctioned, and the animal was extubated. Blood glucose, blood gases, and pH were checked finally in the awake, conscious animal before removal of the tail catheters. The animals were allowed access to digestive biscuits and food pellets in the cage postoperatively, and were weighed and examined daily until they were killed after 1 wk of survival. The survival time in the 60-min group was 4–6 days.

Histologic procedure. The rats were killed after 1 wk by the intra-aortic perfusion of 4% phosphate-buffered formaldehyde solution at pH 7.4, warmed to 37°C. This was performed via thoracotomy on the tracheostomized or reintubated, mechanically ventilated rat under 0.8% halothane. Heparin, 90 IU, was given into the left ventricle just before insertion of the perfusion cannula. The descending aorta was clamped,

except in the animals with 60 min of isoelectricity, in which the spinal cord was examined. After a 30-s washout of the vasculature with isotonic saline, 400 ml of fixative was perfused from a height of 2 m, taking approximately 20 min. The brain was allowed to fix in situ, and was removed the following day.

Brains were sectioned coronally into 2.8 mm thick slices and processed in graded ethanol and xylol. After embedding the slices in paraffin and subserial sectioning at 8 μ m, staining was carried out with 0.1% cresyl violet and 1% acid fuchsin fortified with several drops of glacial acetic acid.

For quantification of brain damage, sectioning intervals were adapted to obtain specific standard levels of caudate nucleus, cerebral cortex, and hippocampus (Figure 2).

Quantification of brain damage. Quantification of acidophilic, cytoplasmic neurons was performed by direct visual counting by one of us (R.N.A.) at a magnification of 320 $\times$, using a two-channel laboratory cell counter (Clay Adams, Parsippany, New Jersey). These dying neurons were visible with the acid fuchsin stain as bright red masses of cytoplasm often containing intensely red-staining nuclei. The acidophilic cells were established as dying cells by their disappearance and removal from the brain in separate experiments allowing 2 wk and longer of survival.

Normal neurons had the typical "owl's eye nuclei" with clear nucleoplasm and a prominent nucleolus, surrounded by basophilic cytoplasm containing Nissl substance. Due to shrinkage of the dying cells during necrosis, the average diameter of the acidophilic neurons approximated the average diameter of only the nucleus in the remaining normal neurons, being approximately 20–30 μ m in each case. Thus, nuclei only were counted in the normal neurons, but red, acidophilic nuclei or cytoplasm were counted in the dying cells. However, small fragments of acidophilic axonal or dendritic processes, even if in the process of being engulfed by macrophages, were not counted. Since the Abercrombie correction factor¹¹ for counted objects of unequal size would be roughly identical for normal and dying neurons, when counted as roughly similar sized objects in this way, each of the raw cell counts was taken as is for statistical purposes.

The hippocampus was quantified at seven levels throughout its septo-temporal extent (Figure 2A), but due to the extreme antero-posterior level sensitivity of cell counts and the large number of damaged cells at level 1, this level was not used in the statistical analysis.

The caudate nuclei were quantified at the level of the septal nuclei at their widest point (Figure 2B). Two adjacent,

TABLE 1
Physiologic parameters during isoelectricity

Group	No. of animals	PO ₂ (mm Hg)	PCO ₂ (mm Hg)	pH	MABP* (mm Hg)	Temp. (°C)	Blood glucose (mM)
δ -Waves	5	96 $\pm$ 12	38 $\pm$ 2	7.39 $\pm$ 0.01	130 $\pm$ 18	37.2 $\pm$ 0.2	0.84 $\pm$ 0.18
Burst-suppression	2	94 $\pm$ 4	40 $\pm$ 2	7.36 $\pm$ 0.01	160 $\pm$ 5	37.0 $\pm$ 0.1	0.45 $\pm$ 0.18
10 min	7	97 $\pm$ 21	38 $\pm$ 8	7.35 $\pm$ 0.05	151 $\pm$ 23	37.1 $\pm$ 0.2	0.66 $\pm$ 0.27
20 min	6	108 $\pm$ 13	32 $\pm$ 3	7.36 $\pm$ 0.05	151 $\pm$ 7	37.1 $\pm$ 0.1	0.54 $\pm$ 0.17
30 min	6	93 $\pm$ 24	37 $\pm$ 4	7.38 $\pm$ 0.05	158 $\pm$ 9	37.3 $\pm$ 0.3	0.53 $\pm$ 0.17
40 min	6	101 $\pm$ 5	34 $\pm$ 2	7.38 $\pm$ 0.06	150 $\pm$ 9	37.4 $\pm$ 0.4	0.58 $\pm$ 0.27
60 min	6	92 $\pm$ 3	31 $\pm$ 2	7.35 $\pm$ 0.05	125 $\pm$ 7	37.0 $\pm$ 0.1	0.35 $\pm$ 0.06

All values are given as mean $\pm$ SD

*Mean arterial blood pressure

TABLE 2
Recovery in relation to isoelectricity

Group	Time from glucose reinfusion to extubation (min)	Time from glucose reinfusion to first EEG activity (min)	Maximum postoperative wt. loss (g)
δ -Waves	28 ± 16	$2.4 \pm 0.9^*$	39 ± 16
Burst-suppression	27 ± 2	$9 \pm 3^*$	28 ± 0
10 min	42 ± 17	12 ± 6	39 ± 14
20 min	60 ± 18	18 ± 8	35 ± 14
30 min	61 ± 36	16 ± 12	40 ± 20
40 min	118 ± 62	22 ± 7	40 ± 8
60 min	199 ± 24	66 ± 45	73 ± 14

All values are given as mean $\pm$ SD

*Time for EEG to revert to normal

high-power fields (magnification $320\times$) were counted, located as lateral as possible beneath the white matter.

The cerebral cortex was quantified at the level of the subfornical organ (Figure 2C), from the cingulate cortex above the corpus callosum, over the lateral hemispheric surface to the rhinal fissure.

For the purpose of generating a single number integrating total brain damage for each animal, a crude damage index (CDI) was generated for each brain as follows: in each brain region, less than 10% dead neurons was assigned the number 1, 10–50% dead neurons 2, and more than 50% dead neurons 3. The scores for the individual areas were summed to produce the CDI.

RESULTS

Experimental model. Trauma during intubation initially caused postoperative laryngeal deaths, but after ventral transillumination of the throat by a sufficiently bright fiber-optic light source, intubation under direct vision became possible and this problem was alleviated.

The dose of insulin proved critical, overdosage resulting in profound weakness and respiratory death in spite of normal blood sugar values in the recovery period. Such animals showed no excess of brain damage over the above groups, and, indeed, even control animals that subsequently showed no brain damage became extremely weak if the insulin dose was 30–40 IU/kg.

There was no correlation between the insulin dose and the blood glucose level at which isoelectricity occurred (Table 3). Glucose levels either fell, remained constant, or rose during isoelectricity, but whichever of these occurred had no influence on brain damage. Likewise, the absolute levels of blood glucose did not show any correlation with brain damage.

Recurrent bradyarrhythmias, due to episodic vagal discharges during isoelectricity, were completely blocked by atropine, administration of which was regularly associated with a rise in the pulse from control values of approximately 200 to as much as 500 during isoelectricity.

Control of blood pressure to below 180 mm Hg was critical in preventing pulmonary edema and hemorrhage. Physiologic data pertaining to the isoelectric periods are given in Table 1. Hypoxia, hypotension, or temperature changes can be ruled out as factors contributing to the observed brain damage. Recovery with glucose was regularly associated

with a drop in blood pressure, the depth and duration of the drop being proportional to the speed of administration of the glucose. After several ensuing minutes of hypertension, usually to 160–180 mm Hg and accompanied by tachycardia and a widened pulse pressure, the blood pressure gradually fell to control values of around 100 mm Hg. With recovery



FIGURE 3. Cerebral cortex. Sixty minutes of isoelectricity followed by 1 wk of recovery. Damage is virtually localized to the outer layers, visible as neurons with intense affinity for acid stains, rendered punctate and dark in this black-and-white photograph. A normally inconspicuous portion of the ventricle in the white matter is dilated. Acid fuchsin/cresyl violet. Bar = 500 μ m.

TABLE 3
Brain damage in relation to insulin dose, blood glucose and isoelectric period

Experiment	Insulin dose (IU/kg)	Blood glucose (mM)	Time taken (min)*	Caudate left right (%)§	Dentate left right (%)§	CA1 left right (%)§	Subiculum left right (%)§	Cortex 2-3 left right (cells)¶	Cortex 4-6 left right (cells)¶
30-min †	13	0.68	30	0	0	0	0	0	0
35-min †	19	0.66	34	0	0	0	0	0	0
45-min †	20	1.09	26	0	0	0	0	0	0
45-min †	11	1.51	41	0	0	0	0	0	0
45-min †		1.19	30	0	0	0	0	0	0
60-min †		0.85	45	0	0	0	0	0	0
60-min †	9	1.37	23	0	0	0	0	0	0
60-min †		0.91	51	0	0	0	0	0	0
6-min BS‡	16			0	1.6	0.2	20	4	0
10-min BS‡	22	0.32	2	0	0.08	0	0	0	0
10-min BS‡		0.57	8	0	0	0	0	0	0
10-min	20	0.8	5	0	0	0.05	0	0	0
10-min				0	0.1	0.35	0.5	12	0
10-min	15	0.39	5	75	0	14	89	33	0
10-min				11	0	26	78	0	0
10-min	11	0.36	5	0	0	0.02	0	0	0
10-min		0.39	8	0	0	0.02	0	0	0
10-min	9	0.72	2	0	0.6	0.2	0.7	0	0
10-min				6.8	8	0.3	65	0	0
10-min	9	1.08	2	0	0	0	0	0	0
10-min		1.00	9	0	0	0	0	0	0
10-min	14	0.48	5	0	0	0.44	35	0	0
10-min		0.34	9	0	0	0.67	49	2	0
10-min	14	1.31	4	0	0	0	0	0	0
10-min		0.51	9	0	0	0.23	0	0	0
20-min	11	0.57	11	33	0	0.23	8	0	0
20-min		0.55	17	50	0	0.21	8	5	0
20-min	10	0.42	10	15	2.6	0.55	87	5	0
20-min		0.12	19	7.1	2.8	0.39	36	1	0
20-min	10	0.86	10	49	4.4	0.09	30	4	0
20-min		0.38	19	49	4.0	0.09	30	0	0
20-min	10	0.64	10	56	0	0.07	38	13	2
20-min		0.70	19	64	1.0	0.04	41	2	0
20-min	15	0.49	10	65	6.7	7.8	85	2	0
20-min		0.33	19	73	7.5	13	69	6	0
20-min	13	0.70	10	49	3.1	3.8	77	5	0
30-min				41	3.8	2.5	50	15	0
30-min	12	0.62	15	85	2.8	0.85	47	108	0
30-min		0.61	28	94	3.5	0.55	36	70	0
30-min	17	0.76	15	55	16	58	88	624	9
30-min		0.81	28	91	19	49	86	238	9
30-min	16	0.49	15	67	14	21	81	14	0
30-min		0.43	28	97	28	37	94	423	0
30-min	21	0.72	15	94	13	10	94	107	4
30-min		0.47	27	80	15	2.3	78	21	3
30-min	15	0.58	15	70	23	14	82	70	3
30-min		0.18	28	79	24	13	94	50	0
30-min	9	0.35	15	68	6	1.8	71	6	0
40-min		0.30	28	66	6	2.3	76	10	0
40-min	10	0.69	20	51	25	13	64	395	54
40-min		1.36	36	87	29	16	74	368	54
40-min	10	0.95	20	59	11	6.2	63	30	1
40-min		0.44	36	38	3	5.0	44	6	3
40-min	14	0.29	20	94	12	50	99.5	499	2
40-min		0.50	38	97	12	53	100	492	14
40-min	10	0.22	20	94	18	3.2	79	456	1
40-min		0.29	36	98	11	2.0	83	167	0
40-min	12	0.66	20	96	21	3.8	88	791	4
40-min		0.51	39	99	22	5.2	95	685	1
40-min	10	0.72	20	94	29	0.74	44	274	0
60-min		0.37	38	96	18	1.6	62	301	2
60-min	14	0.34	20	95	70	60	100	1288	19
60-min		0.28	52	94	67	62	100	1215	44
60-min	15	0.49	30	95	70	41	100	1704	20
60-min		0.26	53	88	67	39	100	1559	2
60-min	14	0.37	25	94	78	63	100	2134	24
60-min		0.34	55	97	90	69	100	2084	28
60-min	15	0.34	25	98	45	6	99	2720	15
60-min		0.26	55	99	51	13	99	2849	14
60-min	14	0.39	20	94	61	80	100	3030	33
60-min		0.24	55	93	57	78	100	2728	74
60-min	15	0.35	25	96	64	44	99	2373	218
60-min		0.57	55	95	71	42	99	2680	289

*Time after onset of slow-wave activity for controls, minutes of isoelectricity for 10–60-min groups

†High-amplitude & wave (1–4 Hz) EEG pattern

‡Burst-suppression pattern of EEG

§Percent of total neurons that show acidophilic necrosis

¶Raw cell counts of number of neurons showing acidophilic neuronal necrosis

by glucose injection, the pulse in the atropinized animal fell from over twice normal (400–500) to control values, and other physiologic parameters also showed a tendency to normalize in the recovery period.

Once the model was refined, survival was nearly 100% in all groups up to 40 min isoelectricity, without intensive care consisting of manual watering and feeding. The 60-min group, however, showed approximately 50% mortality, due to apnea in the awake and behaving animal between 8 and 24 h recovery. The mode of death in the 60-min group was stereotyped. After recovery of consciousness, the animal assumed a characteristic position in the corner of the cage: the neck was hyperextended, and the snout was pressed up into the corner of the cage. The forelimbs were weak, but the hindlimbs were strong, and the animal persistently drove itself into this position with the forelimbs flaccid under the thorax.

Postoperative weight loss averaged 28–73 g, the weight nadir occurring around the third day. Body weight and, hence, age in these young animals had influence on neither survival nor brain damage in the range of weights used (310–495 g). Behavioral deficits that were clinically obvious at 1 wk were present only in some animals of the 60-min group. Results of psychometric testing and their correlation with brain damage will be reported separately.

Electroencephalography. The EEG passed through progressive stages of abnormality (Figure 1) beginning as the blood glucose fell to below approximately 2.5 mM. The first stage comprised rhythmic slowing to the θ (4–8 Hz) and then δ (1–4 Hz) range, and an increase in amplitude to over 200 μ V. When this pattern was fully developed, usually after approximately 1 h at a blood glucose of circa 1.0 mM, no other activity could be seen against the rhythmic slow waves.

The next stage of abnormality comprised total suppression of activity alternating with persistent, high-amplitude δ -slowing. This burst-suppression pattern heralded the onset of isoelectricity, and never lasted longer than 20 min.

The EEG often went through the reverse of the sequence seen in the progressive stages before isoelectricity, demonstrating δ -waves of low amplitude followed by recovery (Figure 1). EEG recovery time was proportional to the insult duration (Table 2). Reversion to normal was seen within 2–4 min after glucose infusion in control animals that had only experienced δ -wave slowing. After 10 min of isoelectricity the EEG reverted to normal in 10–60 min, and was accompanied by an awakening animal at that time. After 60 min of isoelectricity, it took over 1 h for any EEG activity to return. The record remained abnormal until extubation (usually 4–12 h recovery) and the awake but stuporous animal demonstrated an EEG with some background slowing.

Neurohistology. Figure 3 shows a typical cortical field containing injured neurons. The latter show avidity for acid dyes, and are rendered bright red and are easily countable with acid fuchsin. These neurons are undergoing advanced breakdown of cell structure, or cytolysis. With still longer recovery periods, they are regularly removed from the tissue sections. Removal entails the assistance of macrophages in some brain regions if the density of damage is great, but occurs as simple and isolated neuronal cytolysis in areas of sparse or moderate damage.

Table 3 gives the duration of electrical silence, the insulin

dose, the blood glucose, and the quantitated brain damage for each animal. From the table, it is apparent that brain damage was absent unless isoelectric periods had appeared in the EEG (burst suppression or frank isoelectricity). No association of brain damage with factors other than the duration of cerebral isoelectricity is seen. Blood glucose values in the control group approximated those in the isoelectric groups, and were often lower. Within each group, blood glucose values varied by factors of up to four, with no effect on brain damage. Three animals in the 10-min group showed virtually no brain damage after 10 min of electrocerebral silence. No rigid sequence of recruitment of the various brain structures is apparent, although the hippocampus tended to be involved before the caudate nucleus or cerebral cortex. The values for caudate nucleus are spuriously high, due to the location of the sampled fields in the lateral, most severely affected portion of the nucleus (Figure 2). Damage tended to be concordant in its severity within the regions of each hemisphere, but asymmetry among hemispheres was seen. Rhombencephalic sections taken at 500 μ m revealed less damage in the cerebellum than in the cerebral cortex.

As a simplified presentation of the data in condensed form, Figure 4 plots integrated brain damage against the duration of cerebral isoelectricity. Although there is considerable overlap between groups, there is a trend to increasing brain damage with increased periods of isoelectricity.

The distribution of brain damage will be thoroughly described elsewhere,⁹ but deserves brief mention here. Surprisingly, it was different from ischemia in that it demonstrated a relationship to white matter and CSF pathways. The superficial cortex was more damaged than the deep cortex (Figure 3). The portions of the subiculum and dentate crest exposed to the CSF were affected first (Figure 5). CA1 and the caudate nucleus showed gradients of damage along the white matter. The cerebellum was damaged around the outlet foramina of the fourth ventricle in all animals of the 60-min group, as were the motor neurons of the thoracic spinal cord (Figure 6).

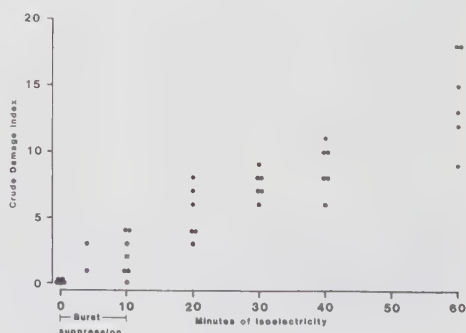


FIGURE 4. A plot of the crude damage index versus minutes of cerebral isoelectricity. The index represents the integrated density of brain damage (see text). Damage is absent unless isoelectricity has occurred. There is considerable overlap between the groups, but damage tends to be worse with increasing duration of EEG isoelectricity.

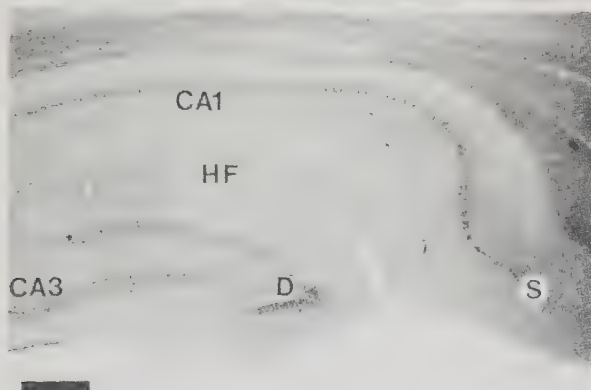


FIGURE 5. Hippocampus, level 5 from Figure 2. Forty minutes of isoelectricity, with 1 wk of recovery. The crest of the dentate gyrus (D), shows dense neuronal necrosis, characteristically affecting a portion of the external blade more than the internal blade. The subiculum (S) is damaged in the open portion exposed to the subarachnoid space, but damage becomes sparse toward CA1 under the closed hippocampal fissure (HF). CA3 is not damaged. Acid fuchsin/cresyl violet. Bar = 500 μ m.

DISCUSSION

The present model was developed from earlier versions used to study clinical behavior, the EEG, cerebral metabolism, oxygen consumption, substrate utilization, and morphology during and shortly after profound hypoglycemia.¹²⁻¹⁷ It was deemed essential to develop a long-term recovery model to allow time for unequivocal neuropathologic changes comprising neuronal necrosis to develop. Pilot experiments with longer than 1-wk survival revealed uniform disappearance of acidophilic neurons from tissue sections; hence, these cells were regarded as necrotic or moribund when observed at 1-wk survival. Performance of the experiments under nitrous oxide anesthesia, with tight control of physiologic variables, was deemed essential to ascribe with certainty the

genesis of these acidophilic neurons to hypoglycemic brain damage and not complicating factors such as hypoxia, epilepsy, or hypotension.

Experimental model. First described by Cannon¹⁸ in 1924, the blood pressure increase and tachycardia associated with hypoglycemia are related to a massive sympatho-adrenal activation that occurs in man¹⁹ as well. The pressor response to hypoglycemia is now known to originate from the brain stem below the level of the inferior colliculi, and to descend in the spinal cord, passing to the adrenal glands via the T5-T7 nerve roots.^{20,21} Cerebral blood flow increases markedly during hypoglycemia¹⁴ and the hypertension may be viewed teleologically as a part of this response. It did not seem to harm the brain in our model: animals not included in the

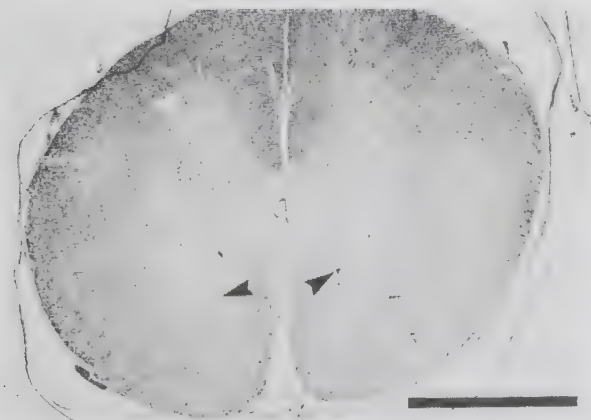


FIGURE 6. Thoracic spinal cord. Sixty minutes of isoelectricity. Several intensely acidophilic motor neurons are seen in the anterior horns (black arrowheads), as well as acidophilic neurons in the nucleus proprius (large white arrow) and substantia gelatinosa (small white arrow). Acid fuchsin/cresyl violet. Bar = 1 mm.

present report, which had a pressor response to over 220 mm Hg, showed only diapedetic microhemorrhages in the thalamus, with a paucity of local neuronal injury and no change in the quantity of brain damage. Extracerebrally, however, the response was harmful to lethal, producing pulmonary edema, pulmonary hemorrhage, and often death during the isoelectricity. In the normotensive animals included in this report, this was not seen, and intensive care measures, consisting of manual watering and feeding of the animals, were required only acutely in some animals of the 60-min group. Small to large cerebral hemorrhages have been seen after short survival in human cases as well.^{22,23}

Blood glucose levels during cerebral isoelectricity were comparable in control and isoelectric animals. There was no direct and simple relationship between the degree and duration of hypoglycemia and the density of brain damage. In this low range, the significance of the absolute level of the blood sugar is dubious. The critical factor determining the onset of brain damage appears to be the appearance of EEG isoelectricity.

Hypoglycemic brain damage. The mechanism of neuronal death in hypoglycemia remains unknown. Although insulin itself was initially felt to be the substance harming the brain, early workers in the field of hypoglycemic brain damage could not demonstrate a direct relationship between the dose of insulin and the resulting brain damage.^{1,2,24}

The absence of brain damage in control rats that were recovered at the stage of large δ -wave EEG slowing indicates that the critical event heralding the onset of neuronal damage is closely related to the onset of EEG isoelectricity. Experiments in animals and in humans voluntarily consenting to undergo hypoglycemia to the point of δ -wave EEG slowing showed focal and reversible neurologic deficits.²⁵ Monkeys and cats with arterial occlusions that do not by themselves result in hemipareses show, with progressive lowering of the blood sugar, a reversible hemiparesis.²⁶ These findings indicate the harmlessness of hypoglycemia to the point of δ -wave slowing, and also the importance of borderline blood flow and blood-to-brain glucose transport in the genesis of reversible functional deficits in the early stages of hypoglycemia. Still, the clinical maxim that patients with suspected hypoglycemia should be given glucose immediately after a blood sugar is taken should, of course, be applied in all clinical situations.

In one study in monkeys,⁶ however, evoked potentials were absent for an average of 57 min in seven animals that still showed no brain damage. Two explanations are at hand. Since evoked potentials (signifying transmission failure) disappear before total electrocerebral silence occurs (signifying depolarization of neuronal membranes), one explanation may be that the EEG isoelectric periods were actually quite short, on the order of 10–20 min, after which serial sectioning would be required to detect brain damage with certainty. Alternatively, the monkey brain may better tolerate a similar period of hypoglycemic isoelectricity than the rat brain.

The critical harbinger of neuronal necrosis in hypoglycemia is demonstrated by the present findings to be an event occurring around the onset of EEG isoelectricity. However the event initiating neuronal death is not perfectly synchronous with the onset of isoelectricity, and may occur either

around or after isoelectricity has developed. This is indicated in some animals by the presence of neuronal death after only burst-suppression of the EEG, without frank isoelectricity, and by the absence of neuronal death after 10 min of isoelectricity in other animals. Isoelectricity in one hemisphere before the other^{26,27} would have been missed in the present experiments, since only interhemispheric bipolar EEGs were recorded. It is possible that the seven asymmetric animals became isoelectric in the more severely affected hemisphere first, but confirmation of this would require two intrahemispheric EEGs.

No significant depletion of high-energy phosphates occurs at the stage of δ -wave EEG slowing, but once isoelectricity occurs, these compounds are depleted rapidly in the tissue.¹² Calcium influx and potassium efflux occur at around this time, preceding or following the onset of isoelectricity by up to several minutes.^{26,27} No single acidophilic neuron was found in the more than 100 subserial sections per brain of the control animals recovered at the stage of large δ -wave EEG slowing. Thus, the metabolic and ionic events surrounding isoelectricity are probably necessary prerequisites for neuronal death in hypoglycemia.

An adaptive metabolic response to hypoglycemia takes place after EEG isoelectricity has developed. Endogenous substrates that are mobilized include glycolytic metabolites, citric acid cycle intermediates, and amino acids.^{12,13,16} Phospholipid metabolism^{27,28} is perturbed. An initial release of free fatty acids occurs, with levels reaching a plateau after the initial 5 min of hypoglycemic isoelectricity.^{27,28} This resetting of metabolic homeostasis²⁷ would seem to facilitate neuronal survival, in view of the present results: a 10-min period of cerebral isoelectricity produced zero to very few dead neurons. Conclusions regarding neuronal necrosis based on metabolic data, without histopathologic confirmation of neuronal necrosis, are clearly hazardous. It is for this reason that some skepticism must be expressed for reports that conclude solely from metabolic data that irreversible cell damage has been incurred.²⁹

Conflicting claims have been made regarding the distribution of hypoglycemic brain damage and anoxic-ischemic or hypotensive brain damage. The distributions have been regarded by some as identical^{15,30–32} and by others as distinctly different.^{4,8} The present results suggest a relationship of necrotic neurons to the CSF. The localization of damage to the superficial cerebral cortex, the crest of the dentate gyrus, the exposed portion of the subiculum, and the cerebellum surrounding the outlet foramina of the fourth ventricle are best explained by a CSF-borne neurotoxin, first hypothesized by Weil, Liebert, and Heilbrunn in 1938 on the basis of their careful histologic observations. The tracer protein horseradish peroxidase, when injected into the CSF selectively labels the dentate crest.³³

Numerous authors have reported spinal cord damage in hypoglycemia,^{34–38} with a delayed clinical onset of lower motor neuron paresis and muscular atrophy^{34,37,38} after hypoglycemic coma. In the present study, the motor neurons of the anterior horn were the commonest cells affected, chiefly in the thoracic, but also in the cervical spinal cord. The forelimb weakness and apnea seen in the present study may be related to this affection of motor neurons.

RELATION OF EEG ISOELECTRICITY TO HYPOLYCEMIC BRAIN DAMAGE

ACKNOWLEDGMENTS

The authors appreciate the technical assistance of Heléne Wilhelmsson in development of the model and performance of the experiments, the expertise of Lille-Mor Lindeström, Marianne Forssén, and Maud Salomonsson in preparation of the sections, the advice of Dr. Arne Brun, Kerstin Stureson, and Elsie Andersson in histologic methodology and for free access to photomicroscopy.

This research was supported by the Swedish Medical Research Council (projects 12X-03020 and 14X-263) and by the NIH (grant no. 5-R01-NS07838). Dr. Auer is the recipient of a Medical Research Council of Canada Fellowship.

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Original Works

The Distribution of Hypoglycemic Brain Damage

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Summary. Rats were exposed to insulin-induced hypoglycemia resulting in periods of cerebral isoelectricity ranging from 10 to 60 min. After recovery with glucose, they were allowed to wake up and survive for 1 week. Control rats were recovered at the stage of EEG slowing. After sub-serial sectioning, the number and distribution of dying neurons was assessed in each brain region. Acid fuchsin was found to stain moribund neurons a brilliant red.

Brains from control rats showed no dying neurons. From 10 to 60 min of cerebral isoelectricity, the number of dying neurons per brain correlated positively with the number of minutes of cerebral isoelectricity up to the maximum examined period of 60 min.

Neuronal necrosis was found in the major brain regions vulnerable to several different insults. However, within each region the damage was not distributed as observed in ischemia.

A superficial to deep gradient in the density of neuronal necrosis was seen in the cerebral cortex. More severe damage revealed a gradient in relation to the subjacent white matter as well. The caudatoputamen was involved more heavily near the white matter, and in more severely affected animals near the angle of the lateral ventricle. The hippocampus showed dense neuronal necrosis at the crest of the dentate gyrus and a gradient of increasing selective neuronal necrosis medially in CA1. The CA3 zone, while relatively resistant, showed neuronal necrosis in relation to the lateral ventricle in animals with hydrocephalus. Sharp demarcations between normal and damaged neuropil were found in the hippocampus. The periventricular amygdaloid nuclei showed damage closest to the lateral

ventricles. The cerebellum was affected first near the foramina of Luschka, with damage occurring over the hemispheres in more severely affected animals. Purkinje cells were affected first, but basket cells were damaged as well. Rare necrotic neurons were seen in brain stem nuclei. The spinal cord showed necrosis of neurons in all areas of the gray matter. Infarction was not seen in this study.

The possibility is discussed that a neurotoxic substance borne in the tissue fluid and cerebrospinal fluid (CSF) contributes to the pathogenesis of neuronal necrosis in hypoglycemic brain damage.

Key words: Hypoglycemia — Cerebral damage — Cerebrospinal fluid — Interstitial fluid — Neuronal necrosis

Introduction

The distributions of hypoglycemic brain damage and ischemic brain damage have long been considered to be identical in the various cortical laminae and nuclei of the brain. Neuroanatomic and, by implication, metabolic differences among different neuronal types [71] have been considered in explaining the distribution of brain damage, as have blood supply differences [63]. Other factors contributing to the distribution have received little attention in discussions on hypoglycemic brain damage, probably because it was assumed that the distribution was identical to that seen in ischemia [15, 16]. Pathologic material from human cases can only be interpreted with the caveat that the damage cannot with certainty be ascribed solely to hypoglycemia, due to the omnipresent specter of possible hypotension, ischemia, hypoxia, or epilepsy, which hypothetically could aggravate or even be solely responsible for the damage.

* Supported by the Swedish Medical Research Council (projects 12X-03020 and 14X-263) and the National Institutes of Health of the United States Public Health Service (grant no. 5 R01 NS07838).
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Experimental studies using short recovery times [3] give an indication of the topography of neuronal injury within the brain, but unless time is allowed for unequivocal pathologic features of neuronal necrosis to develop, it cannot be stated with certainty whether the observed cellular changes representing this injury are reversible or irreversible.

To circumvent the interpretative limitations imposed by clinical material and by short recovery times prior to pathologic examination, a model was developed in the rat, which allowed a pure primary hypoglycemic insult to be delivered to the brain, with long term survival and study of neuronal necrosis 1 week later. The aim was to study the distribution of purely hypoglycemic brain damage both between the two hemispheres and within each hemisphere. Secondary aims were to compare the resulting distribution with that seen in the same species in ischemia, and to analyze the distribution with regard to possible mechanisms of neuronal death. Initial examination of sections revealed the surprising result that although the cerebral cortex and deeper telencephalic gray matter structures were vulnerable to both hypoglycemic and ischemic damage, the distribution within each brain region in hypoglycemic animals was found to be quite different from that seen in a rat model of ischemia [62]. Hence regional quantitative analysis was performed in several brain regions after sub-serial sectioning.

Initially, necrotic neurons were considered to be only those neurons subsequently removed from the brain tissue in separate experiments studying the time course of the tissue damage. However, it soon became apparent that all neurons which demonstrated a pronounced affinity for acid dyes were moribund. Acid fuchsin was found to stain these neurons a brilliant red.

The model itself and the quantitation of whole brain damage in relation to the period of EEG isoelectricity have been presented elsewhere [9]. The present report concerns the distribution of neuronal necrosis in hypoglycemia. The temporal evolution of hypoglycemic brain damage in the rat and correlation of brain damage with psychometric behavioral deficits will each be reported separately.

Materials and Methods

Experimental Model

A previous publication describes more completely the model used [9]. Briefly, male Wistar rats weighing between 285 and 425 g were fasted the night before the experiments, with free access to tap water. Forty to 60 min prior to operation, the rats were given 9–22 IU/kg of regular insulin (Actrapid, Novo Industri A/S, Copenhagen, Denmark). They were immersed in 3% halothane, intubated, and mechanically ventilated with 0.8% halothane in a 2:1 N₂O:O₂

mixture. Two tail veins and the tail artery were then cannulated and continuous paralysis maintained via an i.v. infusion of suxamethonium. Blood pressure was held at 150 ± 10 mm Hg through controlled exsanguination and reinfusion of blood. The arterial PO_2 , PCO_2 , and pH were maintained within the physiologic range. A rectal temperature of 37.5 ± 0.5 °C was maintained by means of a 60 W light bulb 40–50 cm from the semi-prone animal. A bipolar interhemispheric electroencephalogram (EEG) and blood pressure were monitored continuously. After operation, the animal was ventilated on a 2:1 N₂O:O₂ mixture, and cerebral isoelectricity was awaited.

Arterial blood glucose levels during the isoelectric period were determined fluorimetrically using the hexokinase method [30]. After 10 to 60 min of EEG isoelectricity, recovery was induced with an infusion of 0.2 ml of 50% glucose, given by hand over 1 min. A 50% glucose infusion at 1.5 ml/h was then tapered over the ensuing hours and substituted with a 1:1 mixture of 50% glucose and Krebs-Henseleit solution overnight to maintain a plasma glucose level of between 5 and 10 mM/l. Following extubation, usually 1–3 h after glucose induced recovery, blood glucose, blood gases, and pH were checked finally in the awake, conscious animal before removal of the tail catheters. The animals were weighed and examined daily, and they were allowed to survive for 1 week. Separate experiments allowed different survival intervals and greater durations of isoelectricity.

Histologic Procedure

The rats were killed by the intra-aortic perfusion of 4% phosphate buffered formaldehyde solution at pH 7.4, warmed to 37 °C. This was performed via thoracotomy on the tracheostomized or reintubated rat while on a respirator and under 0.8% halothane. Heparin, 90 IU, was given into the left ventricle just prior to insertion to the perfusion cannula. The descending aorta was clamped, except in some animals with 60 min of isoelectricity, where the spinal cord was examined. After a 30 s washout of the vasculature with isotonic saline, 400 ml fixative was allowed to perfuse through from a height of 2 m, taking approximately 20 min. The brain was allowed to fix in situ and was removed the following day.

Brains were sectioned coronally into 2.8 mm thick slices and processed in graded ethanol and xylol. Following embedding in paraffin and sub-serial sectioning at 8 μ m, they were stained with 0.1% cresyl violet and then 1% acid fuchsin fortified with a few drops of glacial acetic acid.

Sub-serial sectioning intervals were adapted to obtain specific standard levels of the cerebral cortex (Fig. 2), hippocampus (Fig. 4), and caudate nucleus (Fig. 8) for regional quantification of brain damage. The hippocampal level chosen corresponds to level 5 from the hippocampal series used for whole brain quantification as described previously [9].

Quantification of Brain Damage

Quantification of dying neurons was performed by direct visual counting by one of us (R.N.A.) at a magnification of $320\times$, using a two-channel laboratory cell counter (Clay Adams, Parsippany, NJ, USA).

Initial pilot studies allowing longer than 1 week survival demonstrated that all acidophilic neurons were removed subsequently from the neuropil through simple cytolysis or phagocytosis. Hence, these were considered either dead or lethally injured in the present study. The possibility of the earliest tinctorial alterations being reversible in the Purkinje cells of the cerebellum is discussed below.

Acid fuchsin stained necrotic cells a brilliant red (Fig. 1). Either nuclei or cytoplasm could stain more intensely acidophilic, and the shape of the nucleus varied from triangular to oval (Fig. 1). Due to

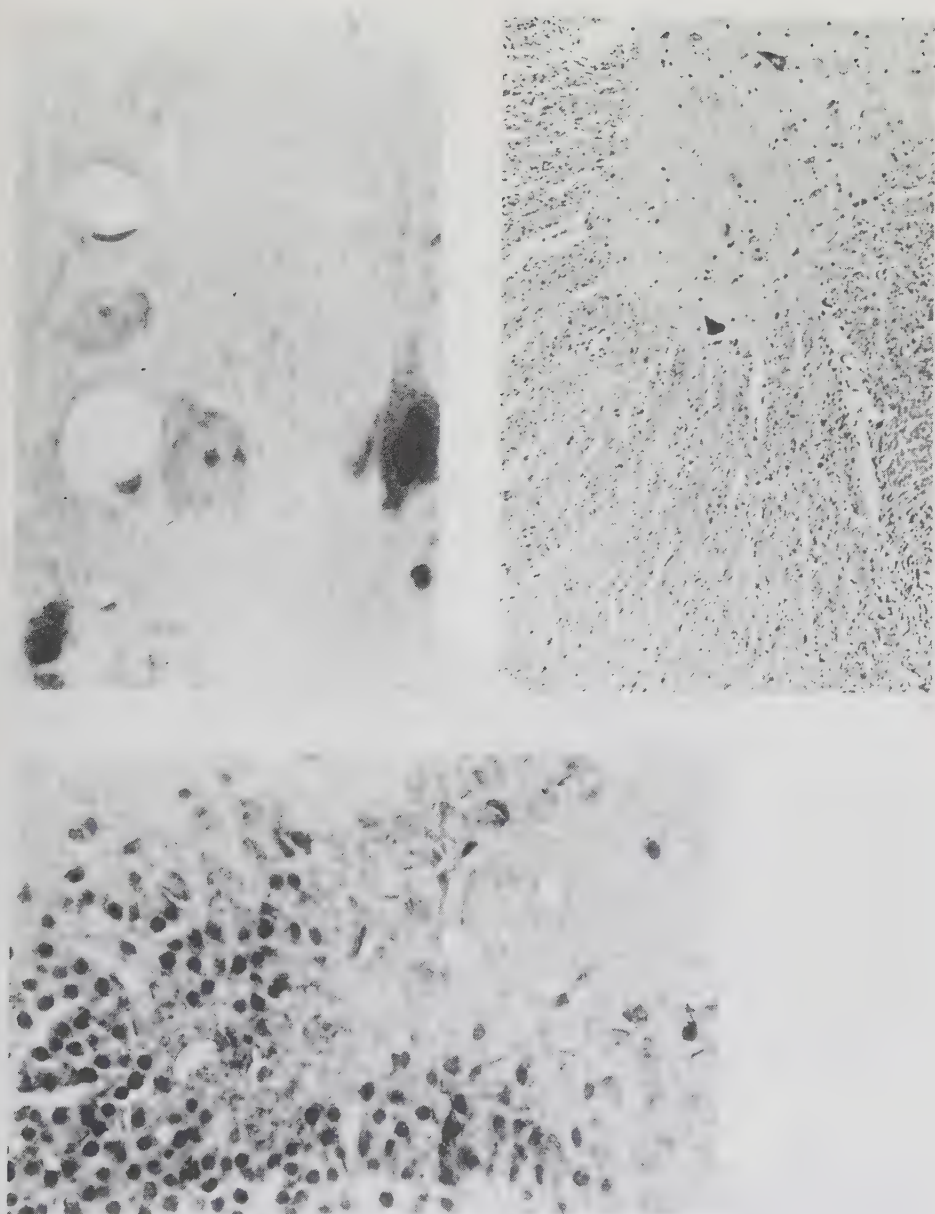


Fig. 1a—c. Appearance of neurons from cerebral cortex (a), anterior horn (b), and dentate crest (c) when stained with acid fuchsin. Either cytoplasmic (a, c) or nuclear acidophilia develops. Nuclei are either round (a, c) or triangular (b). Acid fuchsin/cresyl violet

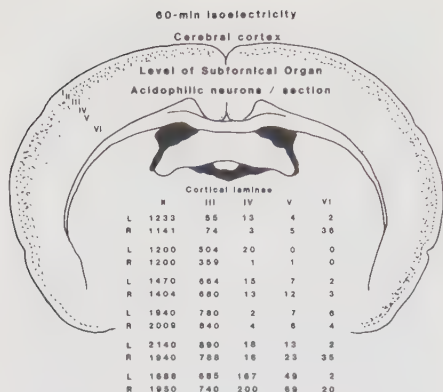


Fig. 2. Schematic diagram of the cerebral hemispheres at the level of the subfornical organ, chosen for laminar cortical quantification. The numerical counts from the six animals with 60 min cerebral isoelectricity are tabled. Although the cortex was quantified from the corpus callosum to the rhinal sulcus, the density of neuronal necrosis is represented schematically over the entire hemispheres. No predilection of damage for the watershed zones is seen after this dose of insult. Damage was sparse in the cingulate gyri (see also Fig. 3)

shrinkage of the dying cells during necrosis, the average diameter of the acidophilic neurons approximated the average diameter of only the nucleus in the remaining normal neurons, being approximately 30 μ m in each case. Thus, nuclei only were counted in the normal neurons, but red, acidophilic nuclei or cytoplasm were counted in the dying cells. Small fragments of acidophilic dendritic processes, even if undergoing phagocytosis, were not counted, however. Since the Abercrombie correction factor [1] for counted objects of unequal size would be identical for normal and dying neurons, when counted as roughly similar sized objects in this way, each of the raw cell counts was taken as is for statistical purposes.

The cerebral cortex was quantified at the level of the subfornical organ (Fig. 2), from the cingulate cortex above the corpus callosum, over the lateral hemispheric convexity, to the rhinal sulcus. In the 60-min group, the individual cortical laminae were counted.

The hippocampus was quantified at seven levels throughout its septotemporal extent (Fig. 4). To assess regional damage within CA1, three microscopic fields were counted individually in the 60 min group, each containing a stretch of pyramidal cells 380 μ m in length. Field A was at the most superior point of CA1, field C was adjacent to the CA1/CA3 border, and field B was midway in between (Fig. 4).

The caudate nuclei were quantified at the level of the septal nuclei at their widest point (Fig. 8). To assess the regional distribution of damage within the caudate, five high-power fields (magnification 320 $\times$) were counted, located beneath the white matter and deep within the nucleus.

Results

Experimental Model

Results pertaining to the animal model, physiologic parameters, and EEG correlation with quantified whole brain damage have been described in detail



Fig. 3. Parasagittal neocortex. Acidophilic neurons are rendered dark and punctate in black and white at this magnification (arrowheads). Note the affection of the superficial cortical laminae over the convexity (narrow arrowheads). There is a relative sparing of the apposed cingulate (CING) gyri. However, their inferior portions exposed to the supracallosal cistern, superior to the corpus callosum (CC), are affected (broad arrowheads). Acid fuchsin/cresyl violet. Bar = 250 μ m

previously [9]. Survival was 100% in all groups up to 40 min isoelectricity, without intensive care consisting of manual watering and feeding. The 60 min group, however, showed approximately 50% mortality, due to apnea in the awake and behaving animal between 8 and 24 h of recovery.

Neocortex

Damage began in the second and third cortical laminae and predominated in these layers after all isoelectric periods. Lamina 2 was clearly more damaged than lamina 3 (Fig. 3). Several of the most severely affected hemispheres showed, in addition, areas of neuronal necrosis in layer 6 and sometimes in layer 5, this pattern becoming distinct in a rat exposed to 2 h of isoelectricity, with 1 day of survival on a respirator.

The superolateral aspect of the hemisphere was always affected first. Increasing cortical damage involved progressively more cortex over the convexity,

both superomedially and inferiorly. After 60 min of isoelectricity, cortical damage extended uniformly from the superior parasagittal region to the inferior surface of the entorhinal cortex. However, the apposed surfaces of the cingulate gyri were relatively spared, except for the inferior portion open to the supracallosal cistern

(Fig. 3). The cortex of the induseum griseum, located below the supracallosal cistern, showed acidophilic neurons as well (Fig. 3).

Hippocampus

The earliest and most consistently affected portion of the hippocampus was the subiculum, followed by CA1 and the crest of the dentate gyrus (Fig. 1c). The medial portion of the subiculum facing the velum interpositum and cisterna ambiens was affected first. Sharp demarcations were seen in the subicular band of cells, between the fused and open portions of the hippocampal fissure. With increasing damage, CA1 became affected, first medially (Fig. 4). A striking gradient was seen along CA1, with the border between affected and unaffected cells located progressively laterally as the amount of damage increased. However, even after 60 min of isoelectricity, the portions of CA1 adjacent to CA3 were spared (Fig. 5). Wallerian degeneration of the axons from the affected septal nuclei was seen in the distal stratum radiatum (Fig. 5).

The dentate gyrus was regularly involved first after 20 min of isoelectricity. At the septal end of the gyrus, where the whole gyrus was open to the subarachnoid space of the velum interpositum, the entire band of cells was uniformly necrotic (Fig. 6). A few sections posteriorly, where increased bulk of the gyrus caused

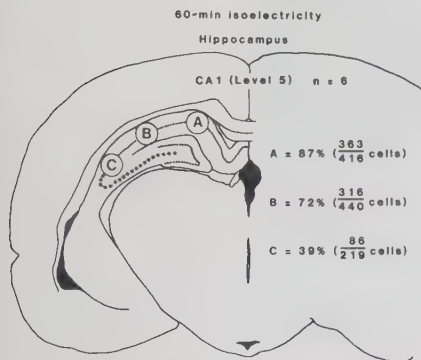


Fig. 4. Schematic diagram of the cerebral hemispheres at the level chosen for regional hippocampal quantification. CA1 is represented by open circles, CA3 by asterisks. There is a gradient of increasing damage as one progresses medially

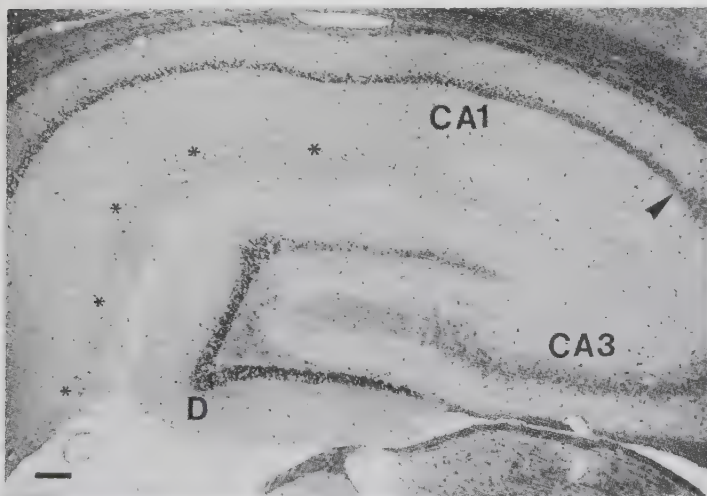


Fig. 5. Hippocampus after 60-min isoelectricity and 6-day survival. There is destruction of the crest and outer blade of the dentate gyrus (D), with a relative sparing of the internal blade. Degenerating neuropil in the dentate hilus, and degenerating mossy fibers (between CA3 lettering and cell band) are acidophilic. The border between CA1 and CA3 is indicated by an arrowhead, and CA1 is spared near the border. Wallerian degeneration of the axons from the septal nuclei is visible in the distal stratum radiatum of CA1 (asterisks), beneath the hippocampal fissure which contains large veins. Acid fuchsin/cresyl violet. Bar = 250 μ m

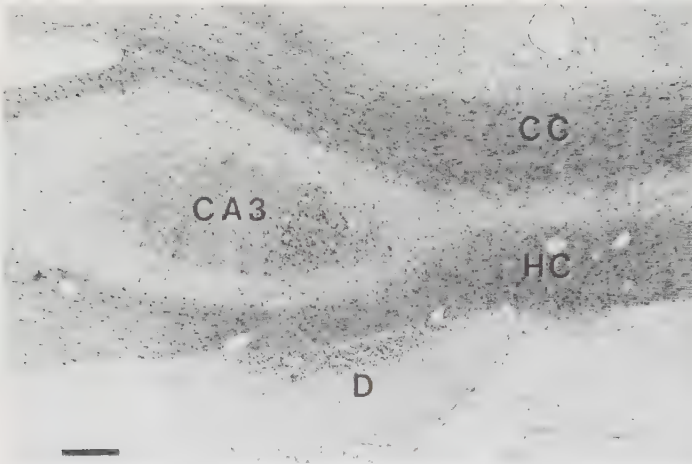


Fig. 6. Septal end of hippocampus, showing CA3, dentate gyrus (*D*), corpus callosum (*CC*), and hippocampal commissure (*HC*), or psalterium. All of the dentate granule cells are necrotic, but there is a sharp border between affected medial CA3 cells and spared lateral cells. The appearance suggests a neurotoxic substance penetrating CA3 medially. Acid fuchsin cresyl violet. Bar = 250 μm

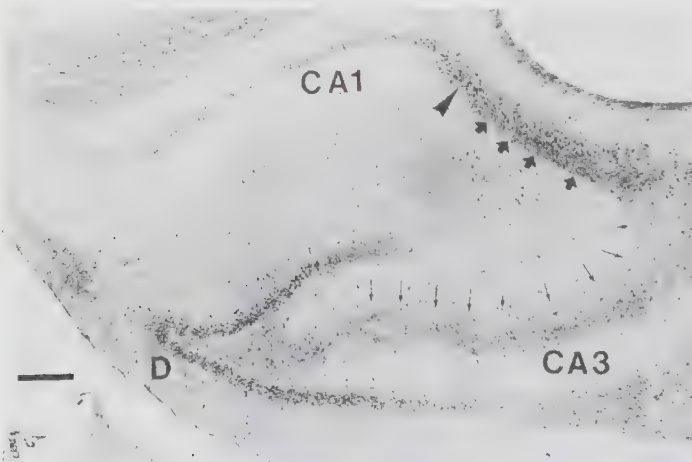


Fig. 7. Hippocampus, in an animal with hydrocephalus. Most of the dentate gyrus is affected, except for the end of the internal blade. There is consequent Wallerian degeneration of mossy fibers which are rendered acidophilic (*thin arrows*). In addition, the portions of CA3 and CA1 beneath the distended lateral ventricle show neuronal necrosis. The proximal stratum radiatum adjacent to affected CA3 is doubly acidophilic due degeneration of apical dendrites as well as mossy fibers (*thick arrows*). The border between CA1 and CA3 is indicated by an *arrowhead*. Acid fuchsin cresyl violet. Bar = 250 μm

apposition of the medial surfaces, the dentate granule neurons were normal beneath the apposed portions of the gyrus. At mid septotemporal levels, the crest of the gyrus was consistently affected first (Fig. 1c), the outer

blade of the gyrus being more affected than the inner blade as damage worsened (Fig. 5). At more temporal levels, the portion of the cell band facing the cisterna ambiens was selectively affected (Fig. 6). CA4 was

involved near the most severely affected portions of the dentate (Fig. 5).

CA3 was the last hippocampal area to be affected. Damage to these cells presented as geographically demarcated areas of acidophilic neurons and acidophilic intervening neuropil (Fig. 6), with the affected side medially. When hydrocephalus was present, damage occurred in the portion of the cell band facing the hydrocephalic ventricle (Fig. 7). Degenerating mossy fibers and apical dendrites both contributed to tissue acidophilia.

Caudate Nucleus

Figure 8 shows the density of neuronal necrosis in each of the five individually counted fields in the caudate nucleus for mildly, moderately, and severely damaged animals. The regional distribution changed with progressively increasing damage to this nucleus, and hence the neuronal counts were pooled into three groups: mild, moderate, and severe. Five of seven rats in the 10 min group showed no pathology whatsoever in the caudate nucleus. In the mildly affected animals, all from the 10 min and 20 min groups, damage was present in the four microscopic fields adjacent to the subcortical whiter matter, most prominently those subjacent to the neocortex (Fig. 8, upper). No acidophilic neurons were seen in the central microscopic field (D) in any animal from this group.

As the damage increased (Fig. 8, middle), the proportion of acidophilic neurons increased markedly in the two lateral microscopic fields (B, C), with lesser damage in the remaining fields adjacent to white matter (A, E). Damage now appeared in the central field of the nucleus (D). These moderately affected animals were seen in the 30 min, 40 min, and 60 min groups.

As the damage increased further (Fig. 8, lower), in a pattern seen in the most severely affected animals whatever the isoelectric period (30 min, 40 min, or 60 min groups), the distribution changed. All these animals showed more severe damage in the superior white matter field nearest the superolateral angle of the lateral ventricle, unlike the less affected animals. Neuronal necrosis was virtually 100% in the field nearest the ventricle in all of these rats. The gradient of damage superiorly was more striking histologically than is apparent from the tabulated neuronal counts, as a dense macrophage infiltration imparted to the tissue a gradient of hypercellularity toward the superior portion of the nucleus and near the white matter (Fig. 9). Numerous normal neurons still persisted in the central field (D). The subependymal portion of the caudate devoid of bundles of white matter (Fig. 10) showed no necrotic neurons, even in the most severely affected nuclei with macrophage infiltration (Fig. 9).

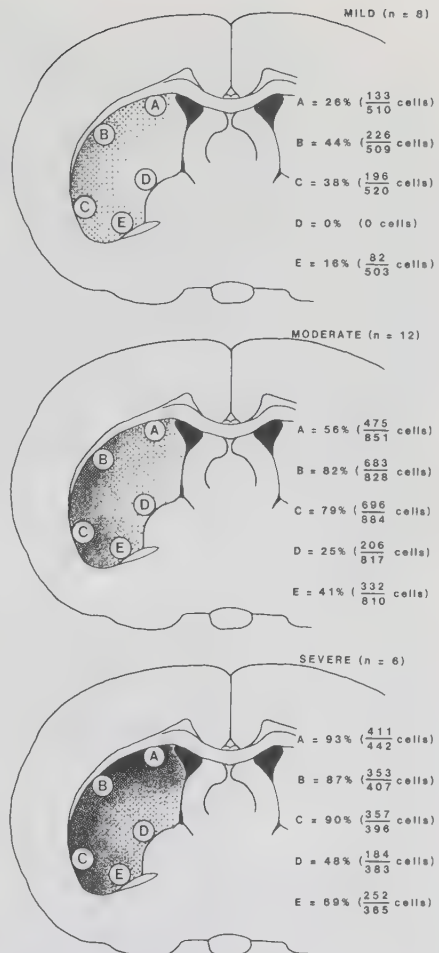


Fig. 8. Schematic diagrams at coronal level (*widest point of septal nuclei*) chosen for regional quantification of caudate nucleus. The location of the counted fields are given on the *left*, and the corresponding cell counts on the *right* for brains with mild (*upper*), moderate (*middle*), and severe (*lower*) damage. The shifting gradient from lateral to vertical argues against a neuroanatomic or vascular basis for the damage. Corresponding histology pictured in Fig. 9 and 10

Remaining Telencephalon

The periventricular amygdaloid nuclei were selectively affected, the lateral and basolateral nuclei [52] containing patches of acidophilic neurons subjacent to the

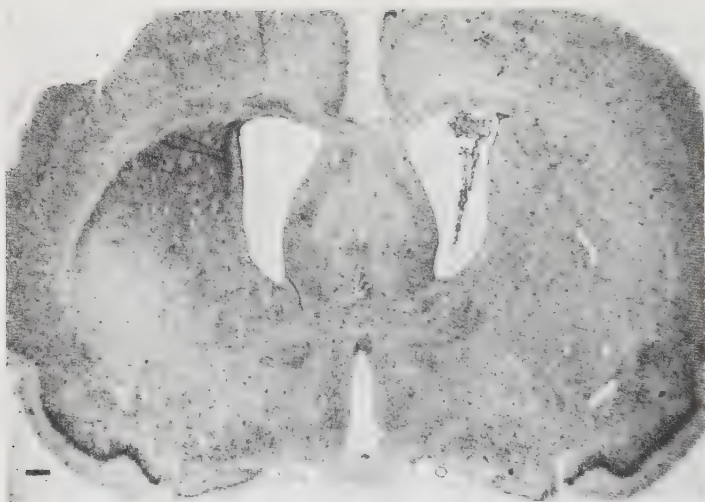


Fig. 9. Animal showing asymmetric damage, 1 week after 30 min of isoelectricity. The caudate nucleus on one side demonstrates severe damage, seen as a dense gradient of infiltrating macrophages. Damage is worse near the white matter laterally and near the angle of the lateral ventricle superiorly. The subventricular zone is spared. The contralateral caudate shows only moderate neuronal necrosis. Cresyl violet. Bar = 1 mm

ependyma of the lateral ventricle. Rare acidophilic neurons were seen in the thalamus and globus pallidus in animals showing the most severe damage. The septal nuclei contained acidophilic neurons, especially in the 60 min group. A selective vulnerability was seen especially in the olfactory tubercle, with sparing of the adjacent neurons and supraoptic nucleus.

Cerebellum

Although occasional acidophilic Purkinje cells were seen after 40 min of cerebral isoelectricity or less, cerebellar damage occurred in all animals first after 60 min isoelectricity. All animals showing cerebellar damage had acidophilic Purkinje cells adjacent to the foramina of Luschka (Fig. 11). Two animals out of six in the 60 min group, and some animals after shorter isoelectric periods, showed acidophilic Purkinje cells over the surfaces of the cerebellar folia as well. Such neurons were rare in the deep portions of the cerebellum. Individual necrotic neurons were seen in the granule cell layer after 60 min isoelectricity.

Brain Stem and Spinal Cord

Scattered individual acidophilic neurons were seen in multiple nuclei in the brain stem, most often related to vacuolated white matter tracts. The spinal cord was

involved in all animals of the 60 min group. Damage was worst in the thoracic cord. Although the anterior horn cells were most often affected (Fig. 1b), there were acidophilic neurons in the nucleus of Clarke and all regions of the posterior horn as well (Fig. 12).

Hydrocephalus

Moderate to severe hydrocephalus was present in most of the brains. Small, normally inconspicuous portions of the ventricle above the hippocampus were more prominent than in control animals (Fig. 5). In two animals marked hydrocephalus was present 5 and 6 days, respectively, after 60 min of isoelectricity. This did not appear to be due to hydrocephalus ex vacuo. The lateral ventricles had the appearance of distension, and subjacent cell bands of the hippocampus showed neuronal necrosis near such ventricles (Fig. 7).

Symmetry of Brain Damage

Seven animals showed prominent asymmetry of brain damage, concordant among the hippocampus, caudate nucleus, and cerebral cortex in these animals. Such side differences were visible at low power by dense macrophage infiltration on one side, with the other side showing far less damage (Fig. 9).

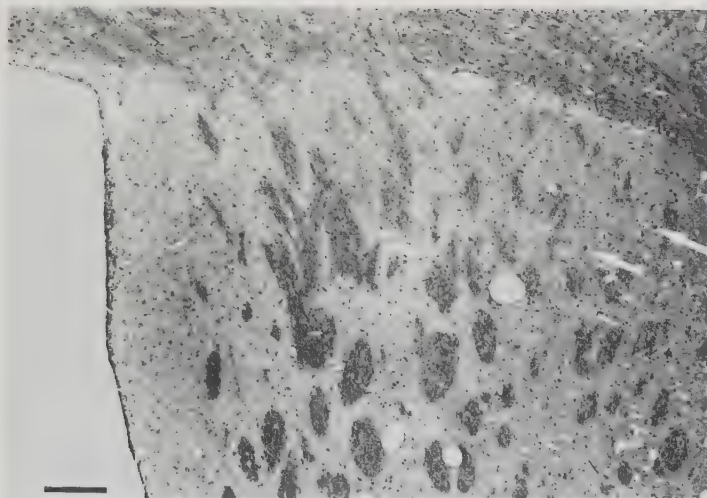


Fig. 10. Caudate nucleus, moderate damage, near the angle of the lateral ventricle. Same animal as in Fig. 9. Necrotic neurons are seen as *black punctate spots*, and are more numerous superiorly and laterally. The large neurons (*white arrows*) are relatively spared. There is no neuronal necrosis in the white matter-free zone subjacent to the ependyma. Acid fuchsin/cresyl violet. Bar = 250 μ m

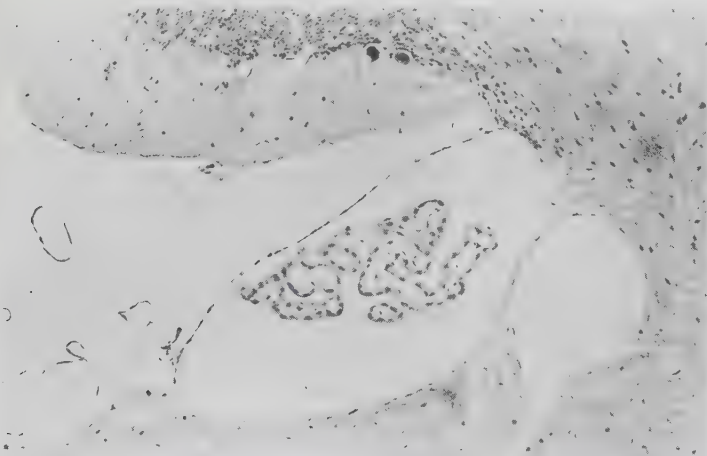


Fig. 11. Cerebellopontine angle near the foramen of Luschka. The choroid plexus protruding from the outlet foramen is seen. Acidophilic Purkinje cells are seen near the foramen. Acid fuchsin/cresyl violet

It is perhaps noteworthy that infarction was not seen in the present study, even with severe damage and 3-week survival. This produced only dense and complete neuronal necrosis, with a vigorous ensuing glial reaction. No cavitation was seen.

Discussion

Although some authors have expressed the opinion that hypoglycemic brain damage and anoxic-ischemic brain damage have a different distribution [10, 49], the

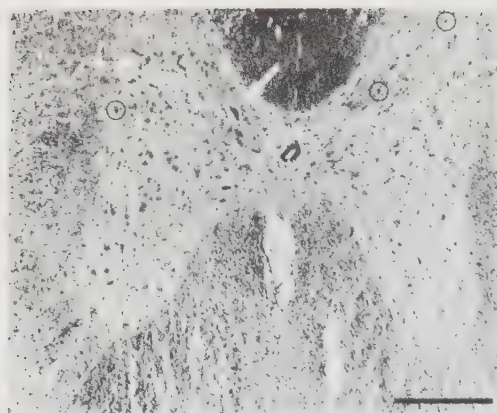


Fig. 12. Spinal cord, demonstrating shrunken, acidophilic neurons in the nucleus dorsalis of Clarke (white arrow), and in the dorsal horns (circled). Acid fuchsin, cresyl violet. Bar = 250 μ m

prevailing view is that the distribution is similar, if not identical [15, 16, 22].

The classic notions of selective neuronal vulnerability are based on the neuroanatomic connections and cytologic features of each brain region, be it a nucleus or a cortex. The logical implication is that the neurons of a given type within the same nucleus or in a similar position within a cortex, having their distinctive Nissl pattern reflecting protein metabolism and their common neuroanatomic connectivities, constitute an anatomic and metabolic unit, and are endowed with a similar selective vulnerability to various diseases.

At first sight, the distributions of hypoglycemic and anoxic-ischemic neuronal damage might be explained by the phenomenon of selective vulnerability and by the fact that both insults lead to similar metabolic perturbations. Like anoxia-ischemia, hypoglycemia of sufficient severity to cause cessation of EEG activity leads to extensive energy failure, cellular depolarization and massive release of neurotransmitters, and to influx of Ca^{2+} into cells, with an associated lipolysis and accumulation of free fatty acids [2, 4, 34, 73]. However, other events are distinctly different in the two conditions. For example, hypoglycemia is associated with an unabated supply of oxygenated blood, with oxidation of cellular redox systems, and with a shift of intracellular pH in the alkaline direction [51, 53]. Many of the neurochemical effects of hypoglycemia must be due to continued oxidation of endogenous carbohydrate and amino acid substrates; notably, this leads to reduction of the tissue stores of many amino acids and to massive accumulation of aspartate.

The present study shows unequivocally that although hypoglycemia affects cells in the classically vulnerable brain regions, the distribution of damage is

different from that in ischemia. This conclusion receives support from a comparison with published data on localization of ischemic neuronal damage in gerbils and rats [37, 42, 54, 62, 67, 68]. Our present results give the surprising finding of a relationship of the neuronal necrosis to the white matter and to ventricular and subarachnoid CSF pathways.

We shall discuss the present results under three headings. First, a review will be given of earlier experimental and clinical studies which were aimed at demonstrating possible brain damage resulting from the then current "insulin shock" treatment for psychiatric illnesses. These data will be correlated to those obtained in the present study, and to results obtained in models of ischemia. Secondly, we shall discuss the possibility that the characteristic features of hypoglycemic brain damage are due to a toxin borne by CSF and tissue fluid, briefly reviewing the current knowledge on CSF and tissue fluid dynamics. Thirdly, we shall speculate on the possible nature of the hypothetical toxin.

Earlier Experimental and Clinical Studies

Only the salient features of those early publications containing adequate pathology will be briefly summarized here, with reference to their common themes in the distribution of hypoglycemic brain damage. This distribution will be compared to that seen in the present study for the major regions of the CNS.

Cerebral Cortex. Traditionally, ischemia is considered to affect pyramidal cells in laminae 3 and 5–6 [16, 54]. At first sight, neocortical damage due to hypoglycemia has a similar distribution [16]. However, a superficial

distribution of hypoglycemic damage in the cerebral cortex has been noted by numerous authors in animal [33, 65] and human [19, 39, 40] material. One infantile case with long term survival even demonstrated secondary calcification selectively localized to cortical laminae 2 and 3 [10]. This superficial distribution and the severe affectionation of layer 2 are characteristic for hypoglycemic brain damage.

Two additional findings should be recalled. First, a perivascular distribution has been striking in some published photomicrographs, with a virtual clearing of neurons from a circular area surrounding cortical vessels ([65], Fig. 4). Second, hypoglycemic cortical damage can be quite asymmetric. Grayzel [3], after administering large doses of insulin to rabbits, illustrated severe neuronal loss (his Fig. 4) in layers 2 and 3 in one hemisphere, with a normal control pictured (his Fig. 5) from the opposite side of the same animal. Finley and Brenner ([29], their Fig. 14) showed a similar pair of photomicrographs, with an affected and a control region, from corresponding portions of the right and left frontal lobes of the monkey. Apposed or touching gyri, and the bases of sulci [66] however, show symmetry in contrast to the asymmetry often seen over the cerebral convexities. Finley and Brenner ([29], their Fig. 24) and Cammermeyer ([19], p. 623) commented on the remarkable symmetry in the apposed occipital lobes. More recently, in a study of protein synthesis during hypoglycemia, a symmetric relative sparing of the apposed parasagittal neocortex was seen [41].

The present study, in which a pure hypoglycemic insult was delivered, corroborates the superficial cortical distribution of the neuronal damage incurred. In fact, in all animals studied, damage to layer 2 markedly exceeded that to layer 3. Furthermore, the number of affected cells in layers 5 and 6 was small, some animals in the 60 min group having either no or only very few acidophilic neurons (Fig. 2). Our results also corroborate the occasional occurrence of unilateral damage, since seven animals demonstrated marked asymmetry, concordant in the cerebral cortex, caudate nucleus, and CA1 zone of the hippocampus.

Hippocampus. This structure is one of the selectively vulnerable regions. Results obtained in rats suggest that cells in the subiculum, CA1, and CA3–4 are also susceptible to ischemic damage [54]. In the gerbil, though, CA1 pyramidal cells were found to be particularly prone to ischemic necrosis [42, 67]. Our own data, obtained in rats, corroborate those observations [62]. Thus, with short periods of ischemia damage was inflicted upon CA1 and CA4 cells, and those in the subiculum, while more prolonged ischemia was required to damage CA3 cells, and even longer to affect dentate granule cells. It is of importance that, with a

moderate duration of ischemia, the whole CA1 sector may become necrotic, with sparing of CA3 pyramids and dentate granule cells.

As in the cerebral cortex, damage in the hippocampus has been consistently present in hypoglycemia, but in a pattern distinct from that seen in ischemia.

Involvement of the dentate gyrus has been found before in experiments with rabbits [72] and rats [38, 75]. Dentate involvement is pictured from human autopsy material in a standard textbook of neuropathology [16, Fig. 2.46], but this is mentioned in neither the caption nor the text. Weil et al. [72] first noticed in 1938 that the portion of the dentate gyrus facing the ventricle was selectively affected, and first hypothesized a CSF borne neurotoxin in hypoglycemia. The consistent involvement of the crest of the dentate gyrus in the rat in hypoglycemia is not explicable on the basis of either its blood supply [6, 23] or its neuroanatomic organization [6, 11, 31, 36, 76], which have been well studied.

The CA1 pyramidal cell band, or Sommer's sector, has been selectively involved in a study on monkeys [49] and another on human autopsy material [46], but all regions of the hippocampus have been involved in other human cases [7, 44] and in experimental material [38].

The present results confirm and extend those previously reported. Notably, dentate granule cells were affected following hypoglycemia of 20 min duration or longer, with the crest of the dentate gyrus showing dense necrosis. Furthermore, the border between affected and unaffected CA1 pyramidal neurons shifted laterally with increasing damage, arguing against a neuroanatomic basis for the demarcation. Finally, when CA3 cells were affected this occurred in the form of sharply demarcated zones near the hydrocephalic ventricles.

Caudate Nucleus. Ischemia is known to affect small to medium sized striatal neurons in the dorsolateral portion of the nucleus, with a well defined frontier between normal and damaged tissue [54]. Although the caudate is neurochemically heterogeneous, with organization into islets of tissue, the acetylcholinesterase poor, peptide rich striosomes [32], this does not correspond to the intrastratial distribution of damage in ischemia.

Selective affection of the small and medium sized pleomorphic neurons of the caudate nucleus seems to be a practically ubiquitous finding following severe hypoglycemia [7, 39, 44]. In the present study, the gradient of damage was positive toward the lateral white matter in all animals. Unlike ischemia, sharp demarcations of damage were not seen, except for the white matter free zone beneath the lateral ventricle (Figs. 9, 10), which was spared. Also unlike ischemia, the less severely damaged caudate nuclei showed a

decreasing gradient along the white matter superiorly, whereas the most severely damaged animals showed an increasing gradient superiorly approaching the ventricle (Fig. 9). This and the shift of the gradient of damage with varying densities of insult speak against a neuroanatomic basis for the distribution of damage seen within the caudate. As in ischemia, the distribution does not correspond to the striosomes [32] of the caudate.

Cerebellum

In hypoglycemia, the cerebellum suffers a less severe metabolic insult than neocortical and limbic areas [2, 5, 55]. This may explain why cerebellar damage is infrequent in the experimental and clinical literature. Nonetheless, autopsy reports in man have noted involvement over the crests of the cerebellar folia [39]. The present study shows that following prolonged hypoglycemia slight cerebellar involvement was present, and with a novel localization. Thus, cerebellar damage appeared first around the outlet foramina of the fourth ventricle. All animals in the 60 min group showed damage around the foramina of Luschka, and two showed damage over the cerebellar hemispheres as well, involving the more superficially located folia. Some of the Purkinje cells demonstrating only minimal acidophilia cannot with certainty be described as moribund at present. This would require a longitudinal temporal study. However, the intensely acidophilic neurons with shrinkage of the cytoplasm, blurring of cytologic details, and degeneration of the dendritic tree can be safely regarded as undergoing necrosis.

Spinal cord. Numerous authors have reported spinal cord damage in hypoglycemia [48, 61, 69, 70, 74], often with a delayed clinical onset of lower motor neuron paresis [48, 61, 70]. In the present study, the motor neurons of the anterior horns were the commonest cells affected, chiefly in the thoracic, but also cervical spinal cord, although necrosis of all neuron types in the cord was seen.

Tissue Fluid Dynamics and Hypoglycemic Neuronal Damage

Recurrent themes in the distribution of hypoglycemic brain damage present in the literature include left-right asymmetry, symmetry around the bases of sulci and between apposed gyri of opposite hemispheres, a superficial cortical distribution, a relationship to the ventricles and subarachnoid cisterns, a perivascular distribution, and a relationship to the white matter. The present study demonstrates these patterns as well. It is difficult to explain solely on a vascular or neuroana-

tomic basis. As stated, one previous group of workers speculated that the localization of hypoglycemic neuronal damage adjacent to CSF pathways was due to toxic substances contained in the CSF [72]. The present results support this notion.

The flow of CSF is classically from choroid plexus through the ventricular system and subarachnoid space into the venous system, and, as has been recently demonstrated, into the lymphatic system through the base of the skull [13, 14, 20]. However, tissue fluid of the brain is produced at the capillary-glia complex [26], and molecules in the labyrinthine, 200 Å wide gray matter extracellular space [17] move by a combination of diffusion and bulk flow [56]. Flow is from gray matter to white matter [21, 43, 64], and around blood vessels, in the Virchow-Robin spaces [20]. Upon reaching the white matter, the mechanism appears to be chiefly by bulk flow, as indicated by tracers of widely varying molecular size and charge [43, 75]. Although some resorption must occur on the venular side of the microcirculation in accordance with the Starling forces, a net flow of superfluous fluid into the CSF, analogous to peripheral lymph formation, is likely. In the present context, it is of importance that the cerebral white matter is known to selectively transmit tissue fluid much more easily than the gray matter.

These two systems, the interstitial fluid system and the CSF system, are parallel, but not completely isolated from one another [25]. The tissue fluid of the brain communicates with the CSF, and vice versa, due to the lack of a brain-CSF barrier [12]. Radiolabeled albumin introduced into the subarachnoid space enters the superficial cerebral cortex over its entire convexity [45]. Tracer substances introduced into the lateral ventricle in hydrocephalus label the white matter near the angle of the lateral ventricle [47]. Horseradish peroxidase (HRP) injected into the subarachnoid space labels the outer blade and crest of the dentate gyrus selectively [27]. As HRP is actively taken up and transported by dentate granule cells, both intracellular and extracellular routes of spread of CSF borne substances should be considered. The intracerebral patterns of tracer uptake from CSF in the above mentioned studies resembled the intracerebral patterns of damage in the present study.

The pathologic course of intracerebral tissue fluid has not been studied in hypoglycemia, although several models associated with either an intact or an open blood-brain barrier have demonstrated a flow of gray matter tissue fluid to the subjacent white matter [35, 43].

From the CSF, tissue fluid is free to enter the brain parenchyma [28] via the Virchow-Robin spaces, with unimpeded access to the intercellular spaces of the gray matter, including synapses [18]. Even neuronal uptake of horseradish peroxidase tracer occurs [18, 27]. The

potency of a broad array of toxic substances is increased by hydrocephalus, with entry into brain substances by expanded Virchow-Robin spaces giving rise to perivascular inflammatory reactions [8, 58].

Is There a Fluid-borne Toxin?

Given these facts on CSF and tissue fluid dynamics one can speculate that a fluid borne toxin accounts for the distribution of hypoglycemic neuronal necrosis.

Although we recognize the speculative nature of the issue, we find the localization of hypoglycemic brain damage sufficiently suggestive to justify further consideration. Only boundary conditions for the nature of the presumed toxin can be stated from the histologic patterns of damage. The substance must be capable of destroying all neuronal types in the hippocampus and spinal cord, for example. In the hippocampus, the naturally occurring excitatory amino acids, and their structural analogues [24] fulfill this criterion, as intrahippocampal injection of aspartic acid and N-methyl aspartic acid can cause damage to dentate granule cells, as well as CA1 and CA3 pyramidal cells [50]. Also, injection of N-methyl aspartic acid into the rat caudate nucleus destroys choline acetyltransferase and glutamic acid decarboxylase activities, with a potency comparable to kainic acid [57]. Tissue aspartate levels rise dramatically in hypoglycemia [2]. Alternatively, one could postulate the formation of fluid-borne, membrane-active compounds ("ionophores") which cause cell damage by enhancing membrane leakiness. In fact, it cannot be excluded that the hypothetical toxin is a naturally occurring substance, such as Ca^{2+} , the toxicity of which is well documented [59, 60]. Thus, hypoglycemia causes influx of Ca^{2+} from extracellular to intracellular fluids, with a decrease in extracellular Ca^{2+} from about $1.5 \cdot 10^{-3} \text{ M/l}$ to about $0.15 \cdot 10^{-3} \text{ M/l}$. However, since CSF and freshly formed tissue fluid have a tenfold higher Ca^{2+} concentration than extracellular fluid surrounding depolarized cells, accumulation of these fluids would provide a source of Ca^{2+} , causing further Ca^{2+} and enhancement of Ca^{2+} triggered reactions, such as lipolysis and proteolysis.

We wish to emphasize that although the localization of neuronal damage following hypoglycemia is suggestive, we regard the proposal of a tissue fluid borne toxin merely as a working hypothesis, and the nature of the hypothetical toxin as unknown. In view of its potential importance, though, the hypothesis warrants further study.

Acknowledgements. The authors appreciate the technical assistance of H. Wilhelmsson in development of the model and performance of the experiments, the expertise of L.-M. Lindström, M. Forssén, and M. Salomonsson in preparation of the sections, the advice of Dr.

A. Brun, K. Stureson, and E. Andersson in the histologic methodology and for free access to photomicroscopy. The expertise of the staff of the photography department of Lunds Lasarettet is greatly appreciated. Dr. Auer is the recipient of a Medical Research Council of Canada Fellowship.

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Received February 21, 1984/Accepted April 13, 1984

THE TEMPORAL EVOLUTION OF HYPOGLYCEMIC BRAIN DAMAGE.
I. LIGHT AND ELECTRON MICROSCOPIC FINDINGS
IN THE RAT CEREBRAL CORTEX

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ABSTRACT

In the course of a study on the pathogenesis of neuronal necrosis in severe hypoglycemia, the morphological characteristics reflecting reversible and irreversible neuronal lesions were examined as a function of time following normalization of blood glucose. To that end, closely spaced time intervals were studied in the rat cerebral cortex before, during, and up to one year after standardized pure hypoglycemic insults of 30 and 60 min cerebral isoelectricity.

Both the superficial and deep layers of the cerebral cortex showed dark and light neurons during, and several hours after the insult. By electron microscopy (EM) the dark neurons were characterised by marked condensation of both karyoplasm and cytoplasm, with discernible, tightly packed cytoplasmic organelles. The light neurons displayed clustering of normal organelles around the nucleus with clearing of the peripheral cytoplasm. Some cells, both dark neurons and neurons of normal electron density, contained swollen mitochondria with fractured cristae.

Light neurons disappeared from the cerebral cortex by 4 h of recovery. Some dark neurons in the superficial cortex and almost all in the deep cortex evolved through transitional forms into normal neurons by 6 h recovery. Another portion of the dark neurons in the superficial cortex became acidophilic between 4 and 12 h, and by EM they demonstrated karyorrhexis with stippled electron dense chromatin. The plasma membrane was disrupted, the cytoplasm was composed of amorphous granular debris, and the mitochondria contained flocculent densities. These definitive indices of irreversible neuronal damage were seen as early as 4 to 8 h recovery. Subsequently, the acidophilic neurons were removed from the tissue, and gliosis ensued.

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Thus, even markedly hyperchromatic "dark" neurons are compatible with survival of the cell, as are neurons with conspicuous mitochondrial swelling. Definite nerve cell death is verified as the appearance of acidophilic neurons at which stage extensive damage to mitochondria is already seen in the form of flocculent densities, and cell membranes are ruptured.

Our previous results have shown that hypoglycemic neocortical damage affects the superficial laminae, chiefly layer 2. The present results demonstrate that, following the primary insult, this damage evolves relatively rapidly, in the first 4-12 h. We have obtained no evidence that additional necrotic neurons are recruited after longer recovery periods.

INTRODUCTION

Recent experimental investigations in rats have provided new information on the structural alterations in the rat brain caused by insulin induced hypoglycemia (1,4,5,22). Results obtained when the tissue was fixed by perfusion at the end of 30 and 60 min of hypoglycemia with an isoelectric EEG ("coma"), showed two types of neuronal alterations, together affecting more than half of the neuronal population of the neocortex (1,22). One of these types, the light neurons with peripheral clearing of cytoplasm, proved reversible during a 3 hr recovery period, while the second type of neuronal change (condensed dark staining cells) persisted in part of the neuronal population after that period of recovery (1,22). However, the model used could provide no information on the final cell damage incurred. With the advent of a long term recovery model of pure hypoglycemia, it has now been possible to assess both the density (4) and the distribution (5) of such damage following hypoglycemic coma of up to 60 min duration. One interesting feature

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of these studies is that although hypoglycemic coma of 30 min duration produces dark cell changes in all cortical laminae, only laminae 2 and 3 incur irreversible cell damage, as assessed after 7 days of recovery (4). The results suggest that dark neurons which initially have an appearance suggesting grave damage may either progress to death and dissolution, or recover completely.

A previous article in this series focused on the description of the experimental model we have developed, the density of neuronal necrosis in relation to the duration of isoelectricity, and the functional outcome of hypoglycemia of up to 60 min duration (4). In another paper the distribution of neuronal necrosis within the brain and spinal cord was assessed by light microscopy (5), and the pathogenesis of the injury was discussed. The purpose of the present study was to follow the temporal evolution of the light and dark neurons in the cerebral cortex following a hypoglycemic insult. Two main questions were posed. First, we wished to assess, at the light and electron microscopic level, the progression of structural alterations in dying and recovering neurons and in the surrounding neuropil. In pursuing this problem we utilised the unique differential damage seen between the superficial and deeper cortical layers in hypoglycemia. Second, we wanted to provide information on the time of cell death after hypoglycemic coma of 30 and 60 min duration. Both problems studied required careful consideration of criteria for defining cell death with the optical and electron microscope.

The results demonstrate that neither pronounced hyperchromasia ("dark" neurons) nor mitochondrial swelling are indices of irreversible cell damage. Irreversible damage was definable as discontinuities in nuclear and cytoplasmic membranes and by the appearance of mitochondrial flocculent densities. Such irreversible

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damage was seen as early as 6 to 8 h following the hypoglycemic insults. Cells with these ultrastructural features corresponded to the acidophilic neurons seen by light microscopy, and were regularly removed from tissue sections in the following weeks.

Astrocytic and vascular changes were seen in the cerebral cortex. The present article, however, will concentrate on neuronal pathology in the cerebral cortex. As the sequence of astrocytic and vascular changes was identical to that seen in the CA1 sector of the hippocampus, these changes will be described in the subsequent article.

MATERIAL AND METHODS

Animal Model

A previous publication describes more completely the model used (4). Briefly, 46 male Wistar rats weighing between 285 and 425 g were fasted overnight, with free access to tap water. The next day, 40 to 60 min prior to operation, they were given between 7 and 22 I.U./kg of regular insulin (Actrapid, Novo Industri A/S, Copenhagen). They were then immersed in 3% halothane, intubated, and mechanically ventilated on a 2:1 N₂O:O₂ mixture.

Two tail veins and the tail artery were then cannulated, and continuous paralysis was maintained via an iv infusion of suxamethonium. Blood pressure was held at 150 ± 10 mm Hg through controlled exsanguination and re-infusion of blood. The arterial PO₂, PCO₂, and pH were maintained within the physiological range. A rectal temperature of 37.5 ± 0.5 °C was maintained by means of a 60 watt light bulb 40-50 cm from the semi-prone animal. A bipolar interhemispheric electroencephalogram (EEG), and blood pressure were continuously monitored. The operation for attachment of the physiologic monitoring devices lasted roughly 45 min. The animal

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was then ventilated on a 2:1 $N_2O:O_2$ mixture and cerebral isoelectricity was awaited, the latter usually occurring approximately three h after insulin administration.

Arterial blood glucose levels during the isoelectric period were determined fluorometrically using the hexokinase method (15). After either 30 or 60 min of EEG isoelectricity, recovery was induced with an infusion of 0.2 ml of 50% glucose, given by hand over one min. A 50% glucose infusion at 1.5 ml/h was then tapered over the ensuing hours, and substituted with a 1:1 mixture of 50% glucose and Krebs-Henseleit solution overnight, in order to maintain a plasma glucose concentration of between 5 and 10 mmol/l. Following extubation, usually 1 to 3 h after glucose induced recovery, the blood glucose, blood gases and pH were checked finally in the awake, conscious animal before removal of the tail catheters.

With survival periods of 8 h or less, the rats were paralysed with either a suxamethonium drip or curare, and were mechanically ventilated with N_2O until sacrifice under 1% halothane. With longer survival periods, the rats were re-intubated prior to being killed by perfusion as described above. Animals were perfused after hypoglycemia with delta wave EEG slowing only (control), after 10, 20, 30 and 60 min of isoelectricity without recovery, and after 5, 10, 30 and 60 min, 2, 3, 4, 6, 8, 12, 18 and 24 hours, 2, 3, 5, 7, 14, 21 and 42 days, and 3, 6 and 12 months of recovery. One or two rats were examined after each noted survival period.

Light and Electron Microscopic Procedure

The rats were sacrificed under anaesthesia and mechanical ventilation. After thoracotomy and 90 IU of intracardiac heparin, a cannula dripping isotonic saline at 37°C was introduced into the left ventricle and was secured by a silk suture with its tip

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visible in the ascending aorta. The right auricle was then amputated, the descending aorta was clamped, and the saline was run through the cerebral circulation. Clear perfusate returned from the right atrium within seconds. The perfusing solution was switched after 30 sec to 3% phosphate-buffered glutaraldehyde solution at pH 7.35, warmed to 37°C. The brain was allowed to fix in situ, and was removed after 2 h. All brains were uniformly firm. Coronal slices 1.8 mm thick were cut, revealing cut surfaces uniformly yellow and firm throughout. The slices were divided in the midline. One half of the brain was used for light microscopy and the other half for electron microscopy.

For light microscopy, tissue was dehydrated in a graded series of ethanols up to 95%, embedded in a soft, methacrylate polymer (EFL-67, Serva Feinbiochemica, Heidelberg, West Germany), sectioned at 1.5 μ m, and stained with 0.1% cresyl violet and 1% acid fuchsin fortified with a few drops of glacial acetic acid.

For electron microscopy, five superficial and five deep cortical samples were dissected from the opposite hemisphere over the superolateral convexity at the level of the subfornical organ. Sections were post-fixed in OsO_4 , dehydrated in ascending concentrations of ethanol and embedded in Epon 812. Thick sections were prepared from each of the five samples from each animal, and stained with toluidine blue. Of the five thick sections, one or two showing the widest range of neuronal changes were selected for thin sectioning. These were stained with uranyl acetate and lead citrate, and examined on a JEOL JEM 100C electron microscope.

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RESULTS

General Results

Results pertaining to the animal model have been previously published in detail (4). Briefly, EEG isoelectricity was a necessary prerequisite for neuronal necrosis to occur. Hypoglycemic brain damage did not correlate to the blood glucose concentration, nor to the insulin dose. The critical event heralding the onset of neuronal necrosis was the onset of EEG isoelectricity, and there was a strict correlation between the duration of isoelectricity and quantitated hypoglycemic brain damage, defined as numbers of acidophilic neurons (4). The necrotic neuron counts increased with the duration of isoelectricity up to the maximum survivable duration of sixty minutes, regardless of the blood sugar at which isoelectricity occurred (4).

The reason for this is probably that the onset of isoelectricity coincides with energy failure, sudden loss of ion homeostasis, enhanced lipolysis, and other biochemical events which are secondary to ATP depletion and intracellular accumulation of Ca^{2+} (18,34,40). These events occur abruptly at the onset of cerebral isoelectricity.

Therefore, in the present experiments describing reversible and irreversible changes, the standardised hypoglycemic insults pertain to defined period of cerebral isoelectricity, either 30 or 60 minutes.

Survival in the present study was virtually 100% after 30 min of cerebral isoelectricity, with only rare deaths related to blocked airways. Roughly half the animals survived after 60 min isoelectricity.

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General Morphological Findings

Grossly, the brains were firm, and sections were uniformly yellow. No edema was present after any time interval studied.

Microscopic sections were uniformly free of red blood cells, and thus were considered adequately perfusion fixed. No morphologic alterations were visible in brains from rats which had undergone hypoglycemia without isoelectricity, i.e. to the stage of delta wave EEG slowing (4).

Light and dark neurons, as described previously (1,22), began to appear in the cerebral cortex at 10 min of cerebral isoelectricity, becoming more numerous in all cortical layers up to 60 min of isoelectricity (Fig. 1).

Light neurons demonstrated a peripheral clear space, which by EM was found to consist of watery cytoplasm in the periphery, with normal organelles consequently clustered in a perinuclear position, as described previously (1,22).

Dark neurons in any cortical lamina were characterized by varying degrees of contraction of nucleus and cytoplasm, with a non-specific hyperchromasia to acidic and basic stains, as well as to the heavy metals used for electron microscopic staining.

Rapid changes were seen in the recovery period in the light and dark cell alterations in all cortical laminae. Typically, light cells disappeared within a few hours. The dark cells showed dissimilar fates in the superficial and deep cortical laminae. Superficially, the majority of the dark cells transformed into acidophilic neurons, but in the deeper layers, the dark cell change was reversible within 24 h of recovery (Fig. 1). Previous long term recovery studies have demonstrated the presence of irreversibly damaged neurons in layers 2-3, but their virtual absence from layers 4-6 after 7 days recovery following 30 min of hypoglycemia.

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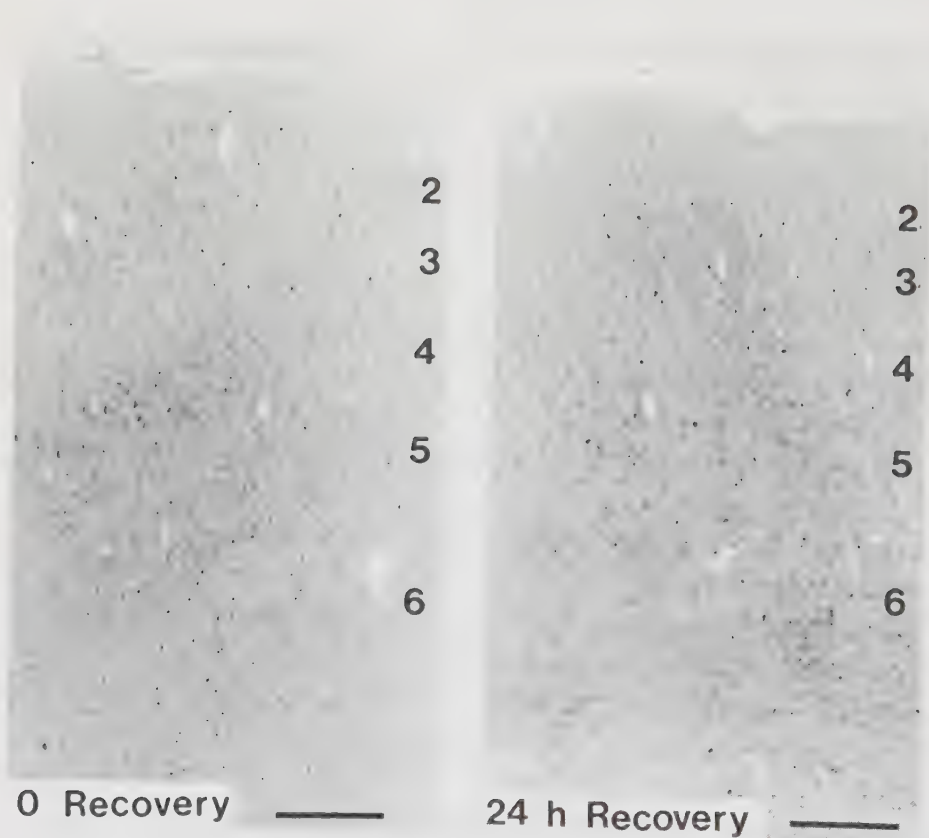


Figure 1. Cerebral cortex at the end of 60 min of cerebral isoelectricity (left) and after 24 h recovery (right). There is recovery of the dark neurons in the deeper cortical laminae, but a transformation of dark neurons into acidophilic neurons superficially. Acid fuchsin/cresyl violet. Bars = 200 μ m.

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Even after 60 min of hypoglycemia irreversible neuronal injury was very rare in layers 4-6 (4,5). Hence, in describing the reversible changes, we use the results obtained from layers 4-6, and illustrate irreversible changes with the sequence of events in layers 2-3.

Cortical Laminae 4-6: Reversible nerve cell changes

Already 4 h recovery abolished the light neuron change after both 30 and 60 min cerebral isoelectricity.

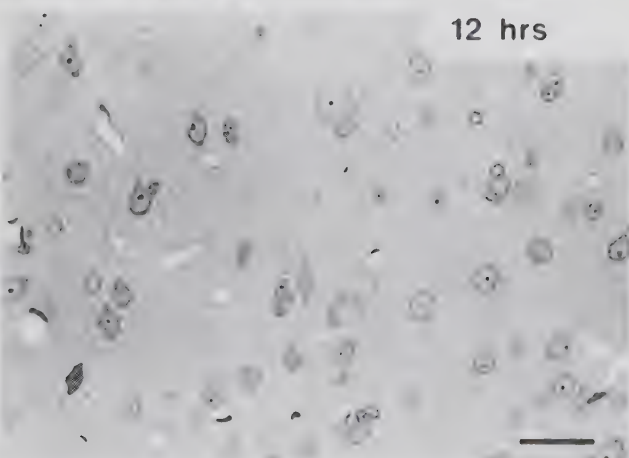
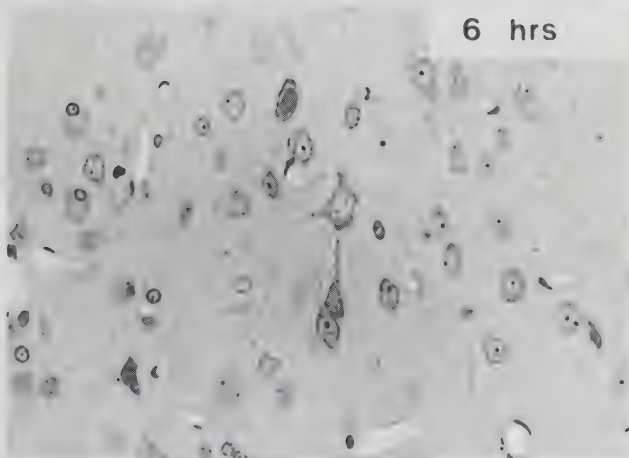
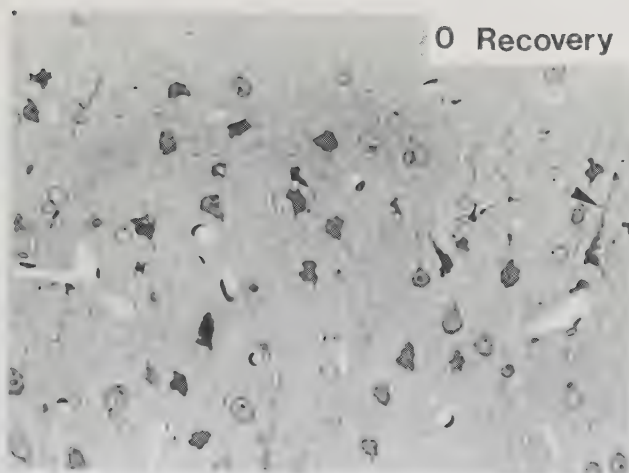
Dark neurons, as observed at the end of 30 or 60 min of hypoglycemic isoelectricity, were contracted, with corkscrew like processes, and were often surrounded by perineuronal astrocytic edema (Fig. 2).

This resulted in an electron microscopic appearance of pronounced electron density, which made the nucleus and cytoplasm often indistinguishable (Fig. 3). Intentional light printing of negatives, however, revealed preservation of organelles and membranes in the hyperchromatic, contracted cells (Fig. 3), compatible with their subsequent recovery in the deeper cortical laminae.

In previous studies on brain tissue fixed by perfusion after 30 or 60 min of isoelectricity, the majority of neuronal mitochondria were normal or contracted, and mitochondrial swelling was conspicuous first during recovery (22). In the present material, inspection of a larger number of sections made it possible to identify neurons with swelling of mitochondria, with loss of internal architecture and fractured cristae. Such mitochondria were seen in both normal and dark neurons (Fig. 4), during cerebral isoelectricity and in the recovery period.

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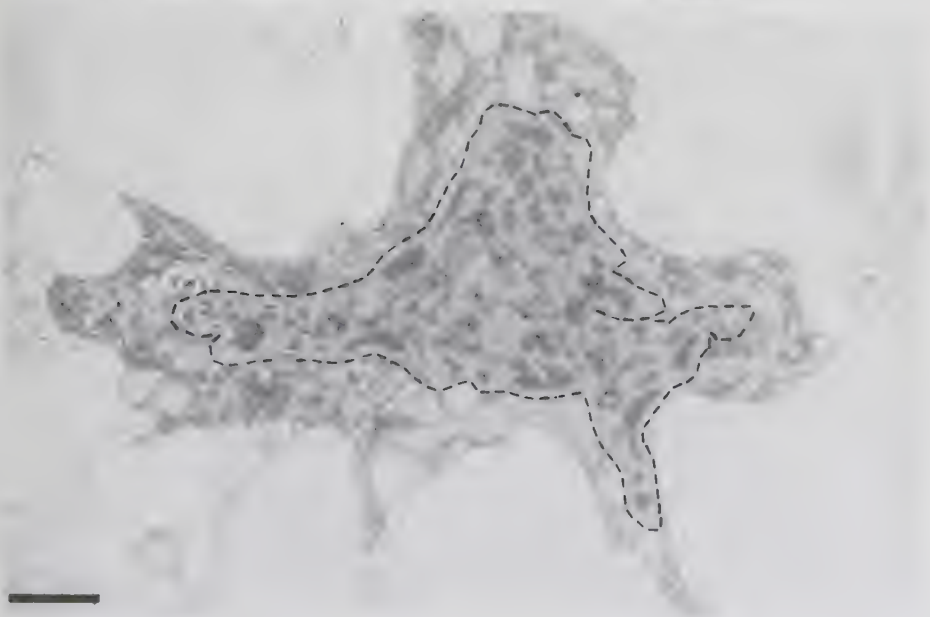
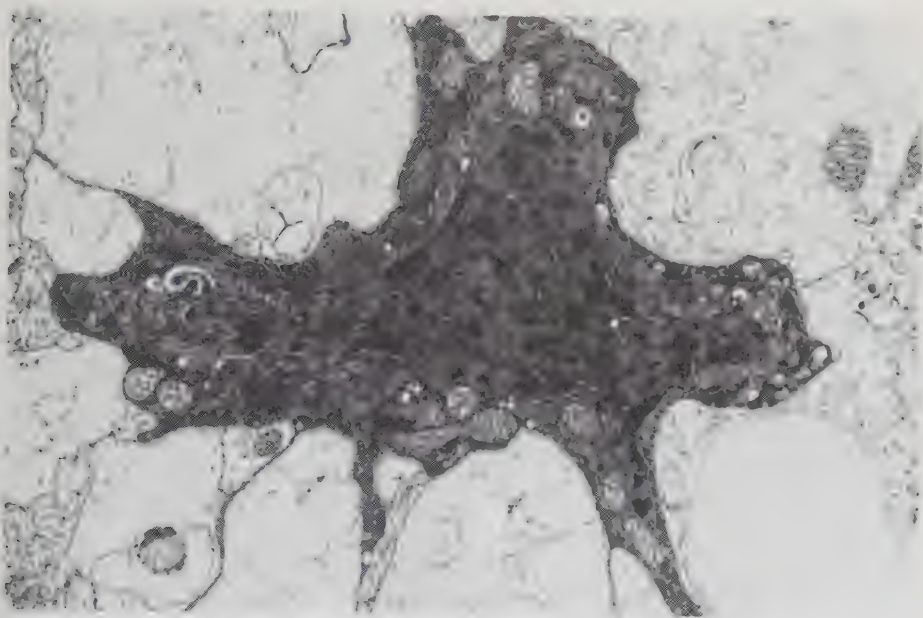
Figure 2 Layer 5 of the cerebral cortex at the end of 60 min isoelectricity, at 6 h recovery, and at 12 h recovery. The dark neurons present during the insult gradually undergo volumetric restoration, and appear normal after one day recovery. Light neurons recover even more rapidly. Acid fuchsin/cresyl violet. Bar = 50 μ m.



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Figure 3. A dark neuron from the deeper cortical layers, after 60 min isoelectricity with apparent loss of internal structure (upper). Intentionally lighter printing however (lower), reveals intact nuclear membrane (inside dashed outline), cytoplasmic membrane, and cytoplasmic organelles, including mitochondria and cristae. Bar = 1 μ m.

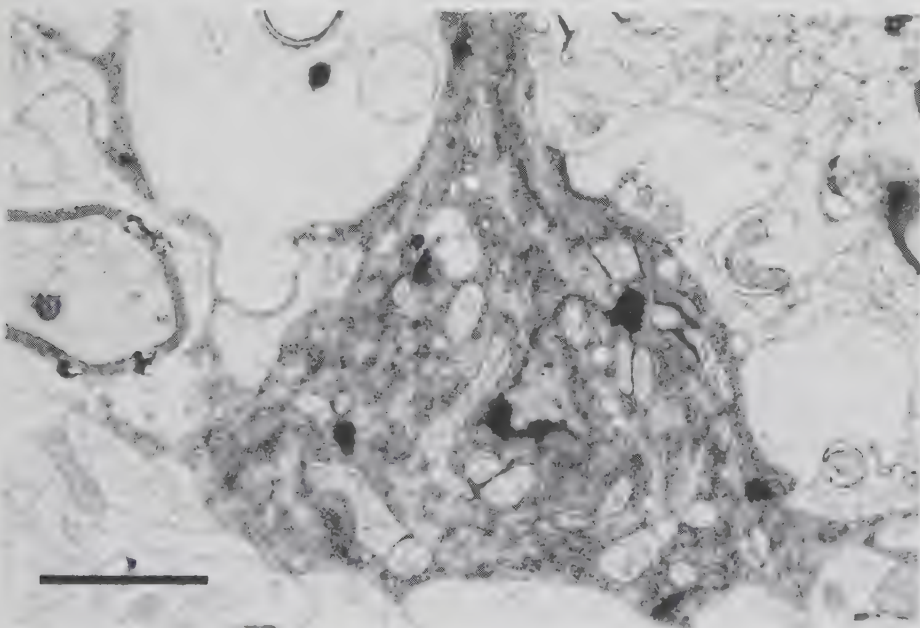
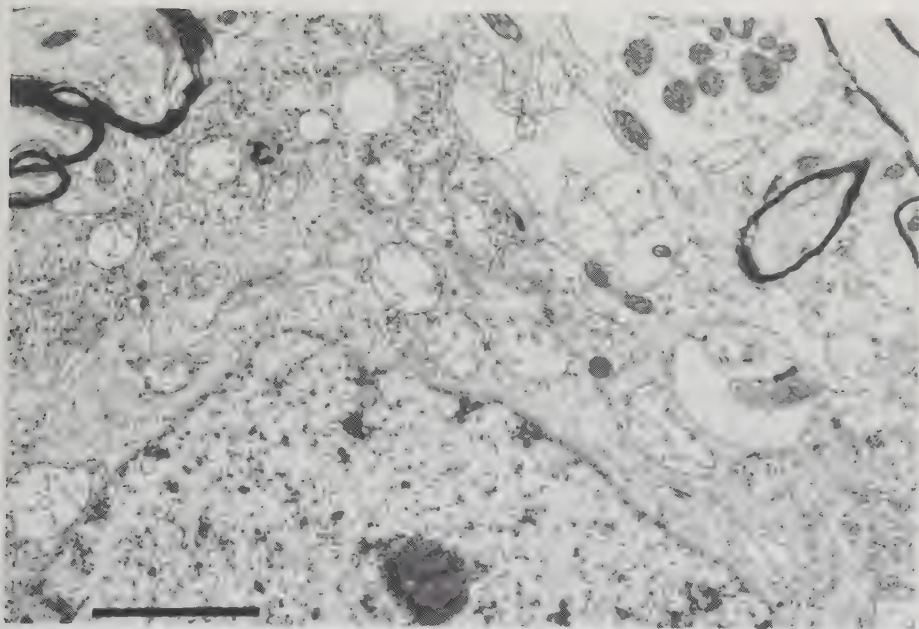
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Figure 4. Neurons from layer 5, at 30 min isoelectricity. Swollen mitochondria (arrows), with disrupted cristae, are seen in both normal (above) and dark (below) neuronal perikarya. Nuclear and cytoplasmic membranes are intact. Bars = 1 μ m.

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The cellular contraction and hyperchromasia of the dark neurons were diminished at 30 min to 1 h recovery, and by 6 h only moderately contracted neuronal forms could be found (Fig. 2). By 12 h, hyperchromasia and volume contraction of cells was minimal to equivocal. These borderline changes resolved completely by 24 h recovery, without the appearance of cellular acidophilia or other tinctorial alteration appearing at any time. No changes were visible by light microscopy thereafter.

Electron microscopy of the recovering neurons in layers 4-6 revealed normalization of the electron density of the nucleus and cytoplasm. Simultaneously, volumetric restoration of the cell occurred (Fig. 5). Subsequently, after 18 h or more of recovery, no definite abnormalities could be demonstrated in neurons of the deeper cortical layers.

Occasional neurons in the superficial and deep cortex demonstrated an increase in the number and size of the Golgi apparatus at 1 to 5 days recovery, with irregular dilatation of Golgi cisternae (Fig. 6). Multivesicular bodies and degenerating mitochondria were prominent in these cells.

Cortical Laminae 2-3: Irreversible nerve cell changes

In layers 2 to 3 of the neocortex, a different picture evolved over time: Many dark neurons underwent transformation into acidophilic neurons (Fig. 7). Most neurons with volume contraction and non-specific hyperchromasia persisted at 2 h of restitution. They demonstrated no specific affinity for acidic or basic dyes, being hyperchromatic to both. By 4 to 6 h, however, a transformation had occurred in the stainability of the cytoplasm, with a selective affinity for acid dyes becoming apparent. This was visualised especially with acid fuchsin (5), and appeared first

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sometimes in the neuronal nucleolus, sometimes in the karyoplasm, but most commonly in the cytoplasm, to persist until lysis of the cell from the tissue during the subsequent days. Cells seem to have acquired cytoplasmic acidophilia suddenly, since no transitional forms between dark cells and acidophilic cells could be found, although dark neurons and acidophilic neurons were seen in the same sections. With the appearance of acidophilia, basophilia diminished, and the cells became extremely difficult to detect by one day in sections stained with only a basic dye (toluidine blue or cresyl violet), where only karyorrhectic nuclear material was seen.

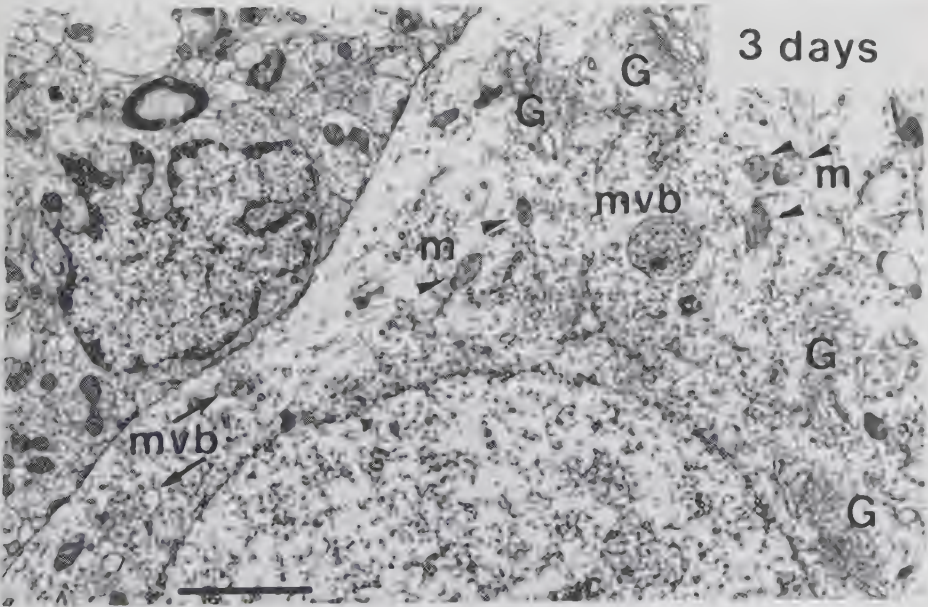
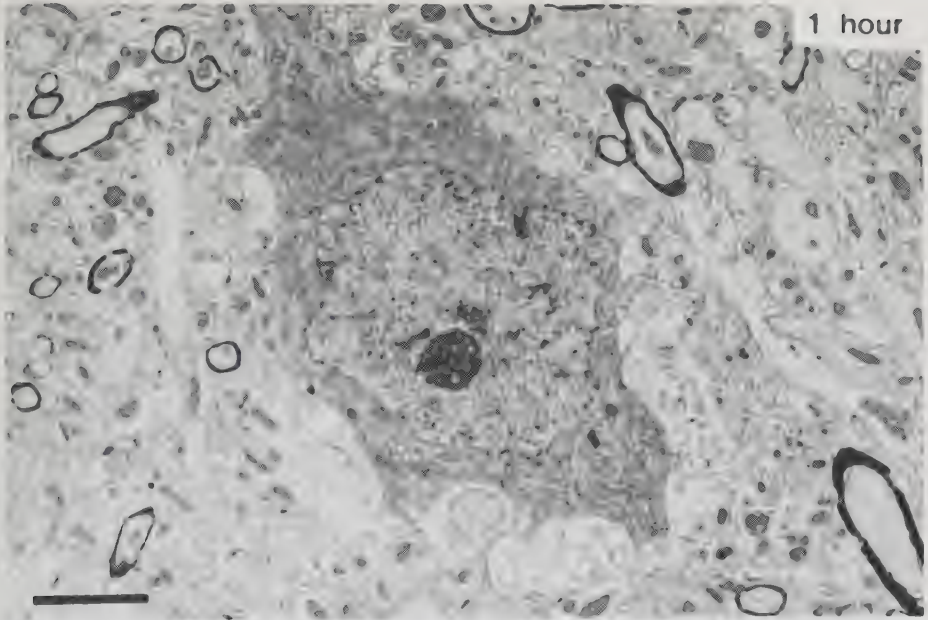
By EM the dark neurons did not show marked progression of the changes during the first couple of hours of recovery. After 4 h, neurons with coarse clumping of hyperchromatic nuclear chromatin and with granular or amorphous gray cytoplasm lacking endoplasmic reticulum or Golgi were commonly observed. Gross discontinuities of the plasma membrane and the nuclear membrane were present, with extrusion of chromatin clumps. Flocculent densities appeared in the mitochondria of such disintegrating neurons (Fig. 8). These cells were considered dead due to their lack of architecture and integrity of cell membranes and compartments. They corresponded in their location, number, and time of appearance to the acidophilic cells of light microscopy, and were the ubiquitous feature of superficial cortical tissue after 6 h. Hence they were considered the electron microscopic counterpart of the acidophilic neuron. Such neurons subsequently underwent complete breakdown of their architecture and phagocytosis by macrophages, producing an appearance identical to that illustrated in Fig. 13 of the first article in this series (23).

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Figure 5. After 1 h recovery following 60 min hypoglycemia, gradual recovery of dark neurons is seen. Volumetric restoration is underway, with resolution of perineuronal edema. Bar = 3 μ m.

Figure 6. A recovering neuron demonstrating prominence and irregular dilatation of the cisterns of the Golgi apparatus (G). Many loose cisternae are not in the orderly stacked arrangement of the normal Golgi apparatus. Degenerating mitochondria (arrowheads) and multivesicular bodies (mvb) are more prominent than normal. Superficial cerebral cortex, 30 min isoelectricity + 3 days recovery. Bar = 1 μ m.

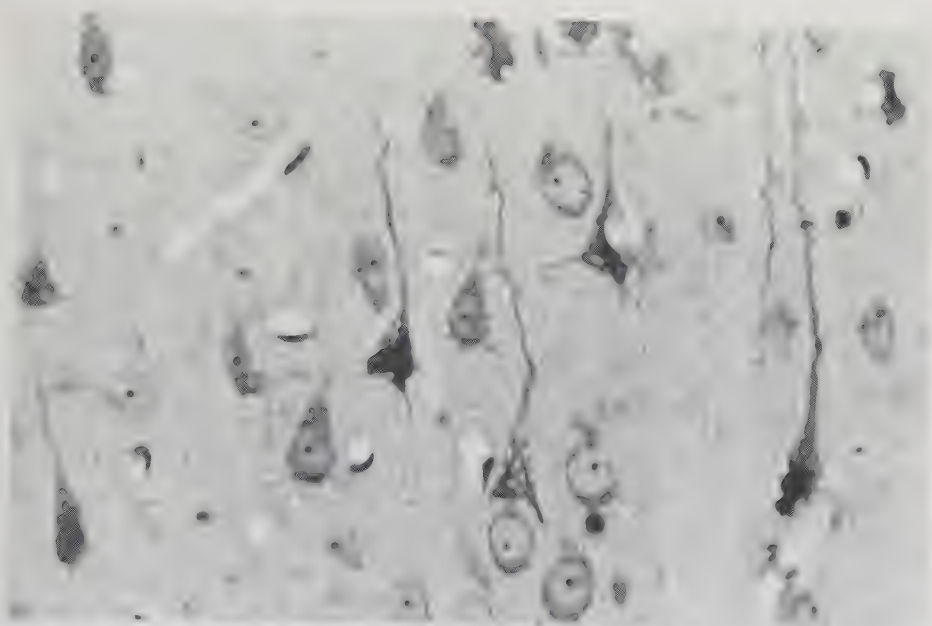
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Figure 7. Dark neuron to acidophilic neuron transformation in layers 2 to 3 of the cerebral cortex. Dark neurons with corkscrew dendrites identical to the cells seen in the deeper cortex, are present at the end of cerebral isoelectricity (above). At one day, only acidophilic neurons are seen (below), which are subsequently removed from the tissue. Acid fuchsin/Cresyl violet.

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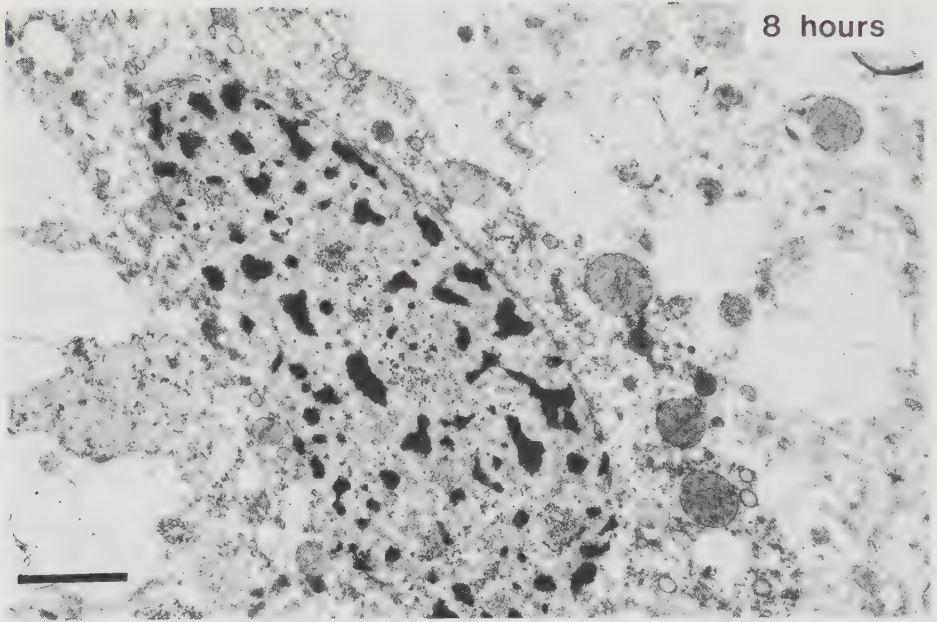


Figure 8. Neuron from the superficial cerebral cortex, with 8 h recovery after 60 min isoelectricity, demonstrating the electron microscopic appearance of the acidophilic neurons of light microscopy. There is admixture of cytoplasmic contents with the extracellular space (arrow) and the nuclear membrane cannot be traced on the left. Flocculent densities are seen in the mitochondria, and the cytoplasm is amorphous. Bar = 1.5 μ m.

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DISCUSSION

A major objective of the present study was to assess the temporal evolution of the dark and light neurons seen in the cerebral cortex during and acutely after hypoglycemic isoelectricity (1,22) and to define a relationship of dark neurons to either reversible or irreversible cellular injury. Dark neurons were shown to revert to normal neuronal forms in the deep cortex, but to progress to acidophilic neurons in the superficial cerebral cortex.

Reversible neuronal changes.

Controversy has surrounded the interpretation of dark neurons seen in the cerebral cortex acutely after hypoglycemia (2,10). It has been recommended that dark neurons be avoided in experimental material (13). A dark and contracted appearance of the perikaryon, with a corkscrew configuration of neuronal processes, has been considered the hallmark of an artefactual change related only to poor fixation (10,13). However, the present findings confirm previous ones (1,22) in showing that such neurons are present in adequately perfused fixed material. In addition, it is demonstrated that in the deeper cortical laminae, dark neuron change gradually reverses through time.

Previous work has shown that 30 min of isoelectricity rarely causes neuronal necrosis in layers 4-6, and that even 60 min of cerebral isoelectricity gives rise to only occasional necrotic neurons in cortical layers 4 to 6 in some animals (4,5). It can thus be concluded that the ubiquitous dark neuron change seen in the superficial and deep cerebral cortex of rats having undergone hypoglycemic isoelectricity cannot be used as an indicator of cell death or a moribund state of the neuron, since almost no neurons in the deeper laminae are destined to undergo necrosis.

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This conclusion is further supported by the present finding of preserved subcellular architecture in dark neurons rendered intentionally lighter through adjusted exposure at the time of photographic printing.

As described above, fractured cristae in swollen mitochondria were seen in some neuronal perikarya, both light and dark. Since such mitochondrial damage was present in the deeper cortical laminae, known to develop only occasional necrotic neurons in large series of rats (4,5), such degrees of mitochondrial injury cannot be considered lethal to the neuron. Furthermore, similar appearing swollen mitochondria, containing disrupted cristae, have been illustrated within neurons in ischemia (16,26,32), status epilepticus (35), alumina cream induced epilepsy (7), and adriamycin toxicity (9), and in all four situations (7,9,33,35) these mitochondria occurred in the setting of non-lethal cellular injury. Although energy production is impaired in hypoglycemia, inhibition of function of mitochondria isolated from whole brain is moderate even after 60 min of cerebral isoelectricity (3).

In all cortical layers, neurons were found which demonstrated changes unlikely to represent a totally catabolic cytoclastic cell. These neurons showed increased numbers of Golgi apparatus, the cisterns of which showed irregular dilatation. Caution must be exercised in the interpretation of this change, since similar irregular dilatation of the Golgi apparatus has also been illustrated in neuronal perikarya in axonal reaction (37, Fig. 16) and in schizophrenia (29, Fig. 1). Swollen and disorganized Golgi seen in rat cortical neurons in ischemia were felt to be an irreversible cellular alteration (32). The present results indicate that this is not the case in hypoglycemia. Our tentative interpretation is that this cellular alteration in the Golgi

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apparatus is a form of non-lethal, reactive cellular change representing increased turnover of internal or external secretory products handled in the Golgi apparatus, perhaps analogous to the reactive hypertrophy of the endoplasmic reticulum described in ischemia (26).

In summary, therefore, neither shrinkage of cytoplasm and karyoplasm with pronounced hyperchromasia, nor marked mitochondrial alteration can be taken to reflect irreversible neuronal damage. In this study, such changes were reversible within the first 6 to 8 h of recovery. Obviously, if neuronal necrosis is to be assessed consequent to hypoglycemia, this is the minimum recovery period which can be employed.

Irreversible neuronal changes

If neither dark neuron change nor extensive mitochondrial alterations can be considered hallmarks of lethally injured cells, it may be asked what marks the neuron as irreversibly injured. A constellation of findings consistently occurring together seems to be the earliest definitive ultrastructural indicator of fatal cellular injury: Amorphous cytoplasm lacking ribosomes and Golgi apparatus, mitochondria containing flocculent densities, thick, electron dense chromatin clumps, large breaks in the nuclear and cell membranes, rupture of cytoplasmic contents into the extracellular space (cytorrhesis), and herniation of nuclear contents into the cytoplasm (karyorrhexis). Neurons exhibiting this constellation of ultrastructural features were universally seen to undergo subsequent cytolysis.

Mitochondrial flocculent densities have been considered a hallmark of irreversible cellular injury (16,19,21,38). This is supported by the present findings, since mitochondrial flocculent

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densities occurred exclusively within neurons exhibiting the ultrastructural features of lethal neuronal injury. Mitochondrial flocculent densities probably represent denatured proteins of the mitochondrial matrix or inner membrane (14).

In several hundred neurons examined in the present study, the above constellation of ultrastructural findings indicating neuronal necrosis was either present in its entirety in a given cell, or was completely absent. It was not possible to find neurons exhibiting part of the constellation of findings. This suggests an extremely rapid and synchronous development of these lethal morphologic changes.

The present results are relevant to the question of whether hypoglycemic brain damage occurs rapidly following the primary insult, or if damage develops gradually, after a morphologic free interval of hours or days, as has been described for CA1 pyramidal cells of the hippocampus of the gerbil (25,26) and rat (27) in ischemia, or in the neocortex of the rat in ischemia (33). The acidophilic neurons of light microscopy, and their electron microscopic counterparts; the karyorrhectic, cytorrhectic cells with mitochondrial flocculent densities, were uniformly present after 8 to 12 h of recovery. Little morphologic change was seen after this time, either by light or electron microscopy, and the acidophilic neurons can therefore be regarded as "cell corpses" awaiting removal from the tissue, either by simple neuronal cytolysis, or with the assistance of macrophages. It must be concluded, therefore, that neuronal death in the superficial cortical layers is relatively rapid, with no demonstrable free interval.

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Pathogenesis of Hypoglycemic Nerve Cell Injury

The question may be posed why only the superficial cortical laminae show irreversible neuronal pathology, whereas the deep cortical laminae show only reversible neuronal damage, after an identical hypoglycemic insult has been delivered to the superficial and deeper cortical laminae. This question relates to the general issue of selective vulnerability of certain brain regions to develop damage, after insults which affect the whole brain.

The cerebral cortex has been shown to sustain superficial damage in hypoglycemia (8,12,17,20,24,30,36), in accord with the present results showing reversibility of acute pathologic changes in the deep cortex. The superficial to deep gradient of neuronal necrosis in the uppermost cortical laminae, revealed by laminar quantification of neuronal necrosis (5), is not explicable on the basis of the neurochemical organization of the cerebral cortex (31).

A previous study has shown the distribution of hypoglycemic brain damage to have a relationship to the surfaces of the brain and ventricles (5). A neurotoxic substance in the CSF has been suggested to mediate hypoglycemic brain damage (5,39), and this may be why irreversible cellular changes develop in the superficial cortex in hypoglycemic brain injury. In support of this, penetration of radiolabelled CSF occurs into the superficial cerebral cortex (28). The present study clearly shows that the dose of insult is much greater in the superficial cortical laminae, the only ones to sustain irreversible neuronal damage. Hence, although definitive evidence must await further experiments, the present pathologic findings are compatible with the hypothesis that neuronal necrosis following hypoglycemic isoelectricity is mediated by a fluid borne neurotoxin. If such a toxin is operative, the

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present results suggest that it mediates cell death within the first few hours of recovery in neurons of the superficial cortical layers.

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THE TEMPORAL EVOLUTION OF HYPOGLYCEMIC BRAIN DAMAGE
II. LIGHT AND ELECTRON MICROSCOPIC FINDINGS
IN THE HIPPOCAMPAL GYRUS AND SUBICULUM OF THE RAT

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ABSTRACT

The previous article has documented the evolution of dark neurons into acidophilic neurons in the superficial laminae as well as the reversion of dark neurons to normal neurons in the deep laminae of the cerebral cortex in hypoglycemic brain damage. The present study describes the temporal evolution of hypoglycemic brain damage in the hippocampus.

The evolution of dark neurons to acidophilic neurons was confirmed in this brain region. Four additional problems were addressed: Firstly, delayed neuronal death was looked for, and was found to occur in areas of CA1 undergoing mild damage. However, it was not preceded by a morphologic free interval, had ultrastructural characteristics distinct from delayed neuronal death in ischemia, and hence should be considered a distinct phenomenon.

Secondly, the gradient in the density of neuronal necrosis in the rat hippocampal pyramidal cell band was exploited to test the hypothesis that a more severe insult causes a more rapid evolution of neuronal changes. This was found to be the case, with a temporal spectrum in the timing of neuronal death: Necrosis occurred already after two hours medially in the subiculum, and was delayed by up to several weeks laterally in CA1.

Thirdly, the almost universal sparing of CA3 pyramidal neurons after 30 min hypoglycemic isoelectricity was exploited to address the question of whether reactive changes, which could with certainty be deemed reversible, occur in CA3. Mitochondrial injury was seen in these cells, and was found to be recoverable. No reactive changes of the type previously described following ischemic insults were observed.

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Fourthly, the astrocytic and vascular response of the tissue was studied. A sequence of astrocytic changes representing structural and, probably metabolic activation of astrocytes was seen, consisting of morphologic indices of increased turnover of cellular components. Capillaries demonstrated endothelial pits, vesicles, and prominent microvilli hours to days after recovery.

The results demonstrate that, in the hippocampal gyrus as in other brain regions, hypoglycemic brain damage is distinct from ischemic brain damage, and likely has a different pathogenesis.

INTRODUCTION

It is well known that the hippocampal pyramidal cells, especially those of the CA1 zone, are vulnerable to both ischemic and hypoglycemic insults (8,13,21,22,23,24,29,34,35,40). In the gerbil, recent studies of reversible ischemia of short duration have shown that the different hippocampal neuronal types react differently to an ischemic insult (9,23,35). Thus, whereas a certain proportion of CA4 cells succumb within the first 8-12 h of recovery, massive necrosis of CA1 neurons occurs first after a free interval of 1-3 days ("delayed neuronal death") (21,22,23,24). The CA3 cells, in contrast, exhibit alterations resembling Nissl's reactive cell change, or central chromatolysis, alterations which are largely reversible (9,23). Slow "maturation" of ischemic neuronal damage has been observed in the rat as well, and shown to encompass both hippocampal and neocortical neurons (24,29,31).

As mentioned in the previous article (4), it has often been stated that ischemic and hypoglycemic brain damage are similar (6,7,8,11,33,39). Our results have shown, however, that this is not the case. Thus, when recovery was instituted for one week following isoelectric hypoglycemia of 10 to 60 min duration, localization of

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neuronal damage differed from that following ischemia (2,3). This also applies to the hippocampus. Neuronal necrosis in the dentate gyrus was conspicuous, with a novel localization to the crest of the gyrus. In the hippocampal gyrus proper, the CA1 and subicular neurons were found not to be equally susceptible along the pyramidal cell band, damage being mild to absent laterally near the border with CA3, progressing to more severe damage medially into the subiculum. Damage was virtually absent from CA3 after 30 min isoelectricity.

The purpose of the present study was to assess the evolution of neuronal and tissue damage in the hippocampal pyramidal ribbon. The first question posed was whether delayed neuronal death, preceded by a free interval of hours or days, occurs following hypoglycemia as well as ischemia. Our second question concerned the rate of evolution of irreversible neuronal damage. The hypothesis has been advanced that the rate of evolution is proportional to the severity of the insult (17). In studying this, we exploited the unique gradient of hypoglycemic damage along the subiculum - CA1 band, assuming that regional variations in the density of cell necrosis reflected corresponding differences in the severity of the insult. A third question studied was whether hypoglycemia, like ischemia, leads to transient changes of the central chromatolytic type, or if the CA3 cells fail to react to the insult both initially, and following long term recovery. Finally, given these regional differences in the density of neuronal necrosis, we posed the question whether reactive changes occurred in glial and vascular elements of the tissue, and if these relate to the density of neuronal necrosis.

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To provide answers to all these questions, we examined tissue of the hippocampal gyrus and subiculum by light and electron microscopic techniques, utilizing samples obtained during 10 to 60 min hypoglycemic isoelectricity, and with recovery of a few minutes to weeks and months. The results provide additional evidence that hypoglycemic brain damage is unique, being distinctly different from that incurred following ischemia.

MATERIAL AND METHODS

Animal Model

A previous publication describes more completely the model used (2). The first publication in this series (4) summarily describes the procedures used in the present experiments, and the material presented here is derived from the same animals.

Forty-six rats were sacrificed after delta wave EEG slowing only (control), and after 5, 10, 30, and 60 min of isoelectricity without recovery with glucose. After recovery with glucose, survival intervals were 5, 10, 30 and 60 min, 2, 3, 4, 6, 8, 12, 18 and 24 hours, 2, 3, 5, 7, 14, 21 and 42 days, and 3, 6 and 12 months of recovery following either 30 or 60 min of cerebral isoelectricity. One or two rats were studied at each time interval.

Light and Electron Microscopic Procedure

Perfusion fixation and histologic processing was performed as reported in the previous article (4). Brains were sectioned coronally into 1.8 mm thick slices, and one half of the brain was used for light microscopy in soft plastic polymer embedding medium (EFL-67, Serva Feinbiochemica, Heidelberg, West Germany). Sections were cut at 1.5 μ m and were double stained with acid fuchsin and cresyl violet.

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The other half of the brain was used for electron microscopy, the sampled areas being indicated in Fig. 1. The hippocampus was dissected out, and five samples each of the medial subiculum and lateral CA1 cell band were taken. Five samples of the CA3 pyramidal cell band were taken roughly halfway between the hilus of the dentate gyrus and the border with CA1. The stratum radiatum of the subiculum was sampled as well. A detailed study of the dentate gyrus will be reported separately (5). After processing in ascending concentrations of ethanol and embedding in Epon 812, thick sections were prepared from each area at each time interval, and stained with toluidine blue. After thin sectioning and staining with uranyl acetate and lead citrate, sections were viewed on a JEOL JEM 100C electron microscope.

RESULTS

Animal Model

Results pertaining to the animal model have been previously published in detail (2), and are summarized in the previous article (4).

Subiculum

After 30 min isoelectricity, light and dark cells were present, as described previously in the cerebral cortex (1,4,20). Swollen mitochondria with fractured cristae were seen in both dark neurons and neurons of normal electron density. Because of the previous suggestion of a CSF borne neurotoxic substance mediating neuronal necrosis due to hypoglycemic isoelectricity, the apical dendrites of the stratum radiatum subjacent to the pia mater were examined from the area indicated in Fig. 1. The dendrites were segmentally swollen (Fig. 2). Already after 10 min isoelectricity, damaged

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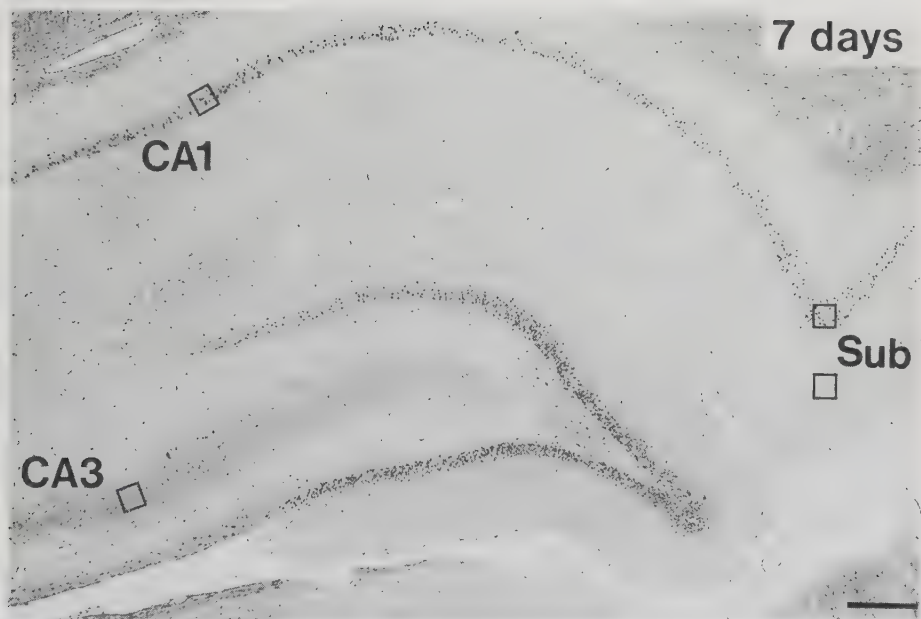


Figure 1. Overview of the hippocampus one week after 60 min of hypoglycemic isoelectricity, with the areas taken for electron microscopy indicated in boxes. There is dense neuronal necrosis in the medial portion of the subiculum, with a tapering density of CA1 neuronal necrosis laterally. No CA3 damage is apparent. Acid fuchsin/Cresyl violet. Bar = 200 μ m.

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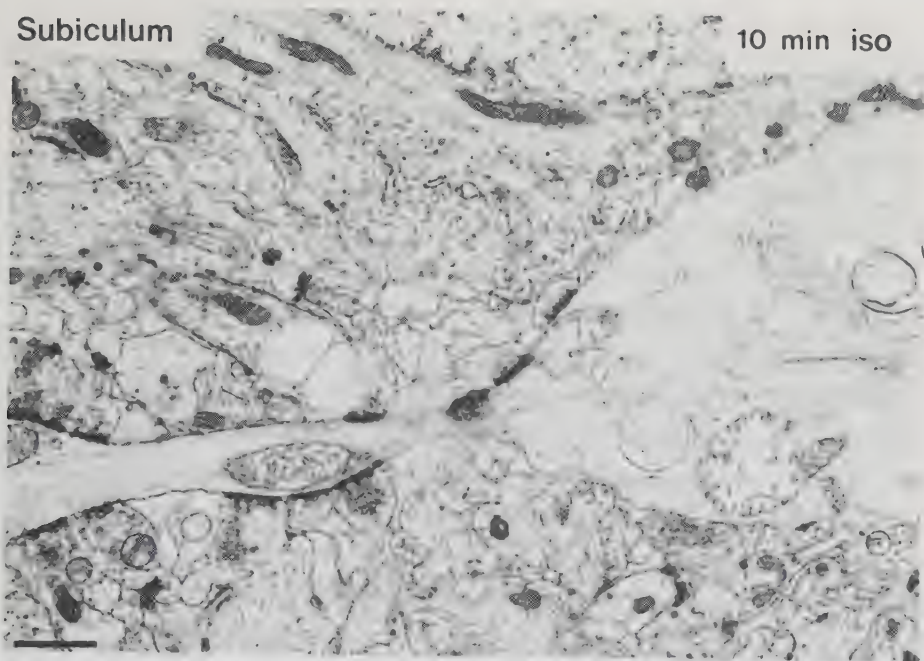
Figure 2. Subiculum after 10 min of isoelectricity. Dendrites of the stratum radiatum are segmentally swollen and contain mitochondria with disordered cristae and varying degrees of swelling. The neuronal perikarya have not yet undergone dark cell transformation at this time. Bar = 1 μ m.

Figure 3. Subiculum two h after 60 min isoelectricity. The characteristic coarse nuclear stippling of chromatin has appeared, along with flocculent densities in the mitochondria and rupture of the plasma membrane. These features together indicate neuronal necrosis. Bar = 1 μ m.

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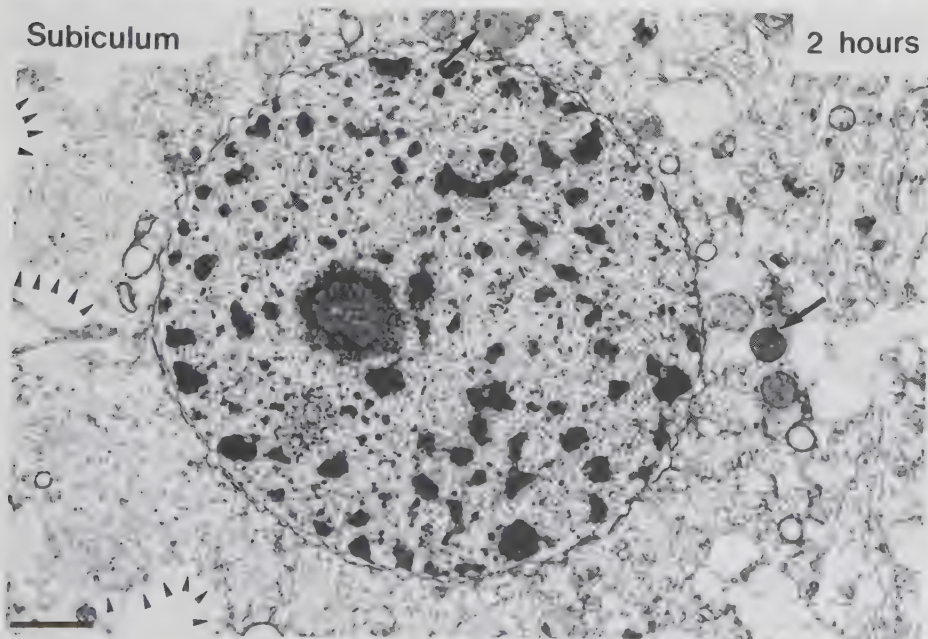
Subiculum

10 min iso



Subiculum

2 hours



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mitochondria were seen in the dendritic processes between the pial surface and the perikaryon. These mitochondria showed fractured cristae, and were often swollen (Fig. 2).

Already after 2 h, a transition to acidophilic neurons had occurred. By electron microscopy, these showed the ultrastructural features of cell death, consisting of mitochondrial dense deposits, amorphous cytoplasm, and rupture of the cell membrane, or cytorrhesis (Fig. 3).

After longer survival intervals than 2 h, little further change was seen in the light or electron microscopic appearance of the acidophilic neurons, until their removal from the tissue by simple cytolysis or through engulfment by macrophages. Neuronal cytolysis in the subiculum was well underway at one week, in contrast to the events seen concurrently in the CA1 neurons (Fig. 4).

CA1

A slower evolution of events was seen in the CA1 pyramidal cells as compared to the subiculum, especially in the more lateral samples near the border with CA3. Medially, nearer the subiculum, the density and time course of neuronal necrosis in CA1 resembled those in the subiculum: During isoelectricity, dark and light neurons were present, the former transforming into acidophilic neurons between 3 h and one day of recovery.

Between one and three weeks, however, neurons were still present in the lateral portion of CA1 which showed preserved cellular outlines with tinctorial changes (Fig. 4). Neurons here showed an affinity for both acid and basic dyes, imparting to them a purple hue. These amphophilic neurons had preserved cellular outlines for one to two wks (Fig. 4), but at three wks, they appeared shrunken and homogeneous.

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Electron microscopy of neurons from these lateral locations, revealed neuronal forms which did not resemble the electron microscopic appearance of the acidophilic neurons. Thus, rather than the coarse stippled chromatin, amorphous cytoplasm, mitochondrial dense deposits, and ruptured cell membrane of the acidophilic neurons, these amphophilic neurons demonstrated homogeneous nuclei, cytoplasm containing recognizable organelles, mitochondria without dense deposits, and preserved cell membranes (Fig. 5, upper). The endoplasmic reticulum of these cells tended to be dilated, as did the Golgi apparatus, with a general increase in electron density. Cell membrane breaks finally did appear, heralding cytorrhesis (Fig. 5, lower).

CA3

The pyramidal cells of CA3 revealed little evidence of even evanescent damage by light microscopy.

By electron microscopy, low power examination revealed an apparent paucity of findings in the CA3 neurons, with only dark degeneration seen in the occasional intervening mossy fiber axons of the dentate gyrus (Fig 6, upper). However, closer examination of the cytoplasm of CA3 pyramidal cells revealed mitochondrial damage (Fig. 6, lower), with fractured cristae and various degrees of swelling. These changes disappeared by one week.

Astrocytic changes

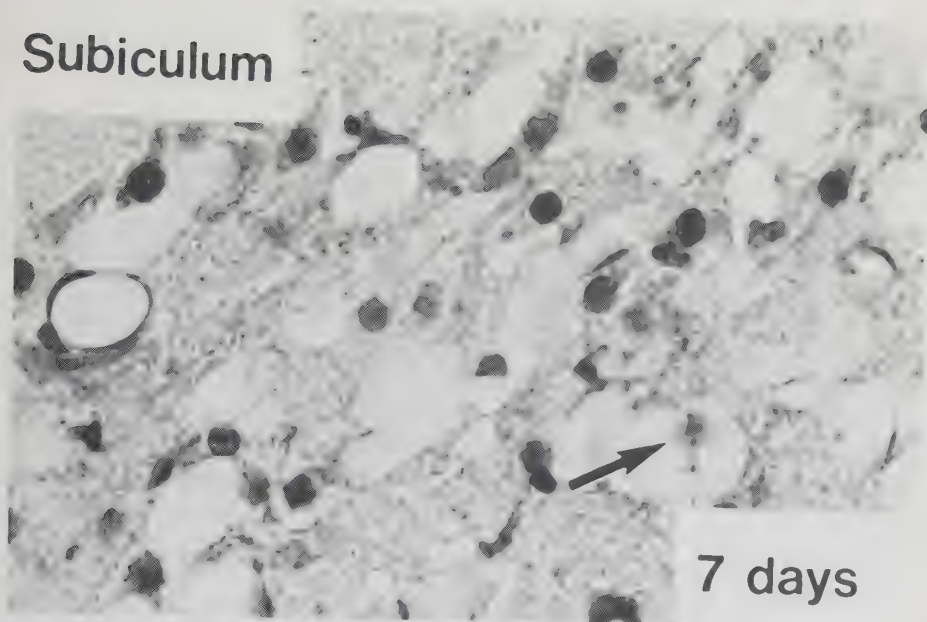
The sequential changes in the astrocytes of CA1 resembled those of the cerebral cortex (4). At early times, e.g. following 12 h recovery, astrocytes showed watery cytoplasm and numerous mitochondria (Fig. 7, upper).

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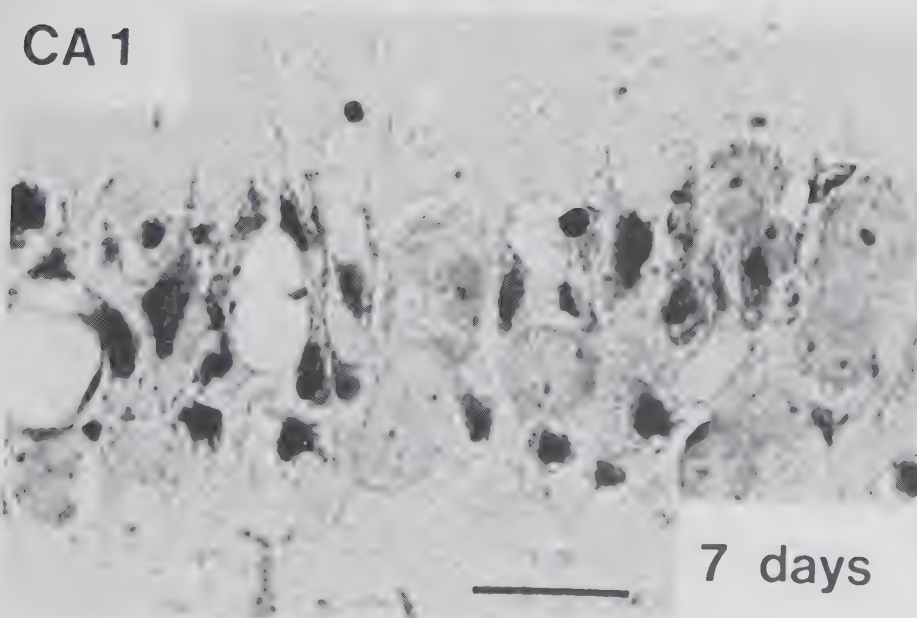
Figure 4. Comparison of the rate of neuronal cytolysis in the subiculum and CA1 at one week. Cytolysis of acidophilic neurons is well advanced in the subiculum, leaving vacuoles in place of perikarya undergoing dissolution (arrow). In the lateral portion of CA1, however, many cells still show preserved nuclear and cell outlines (arrow) and have an affinity for both acid and basic dyes (amphophilia). Acid fuchsin/Cresyl violet. Bar = 50 μ m.

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Subiculum



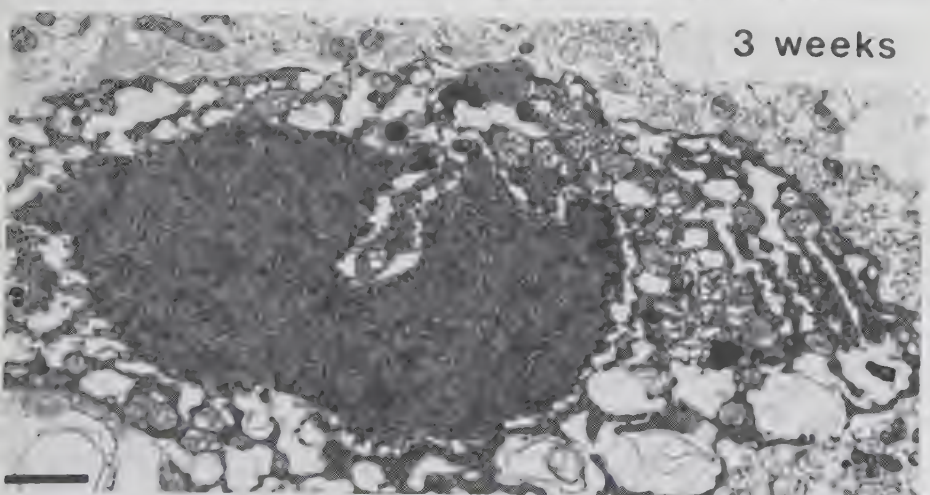
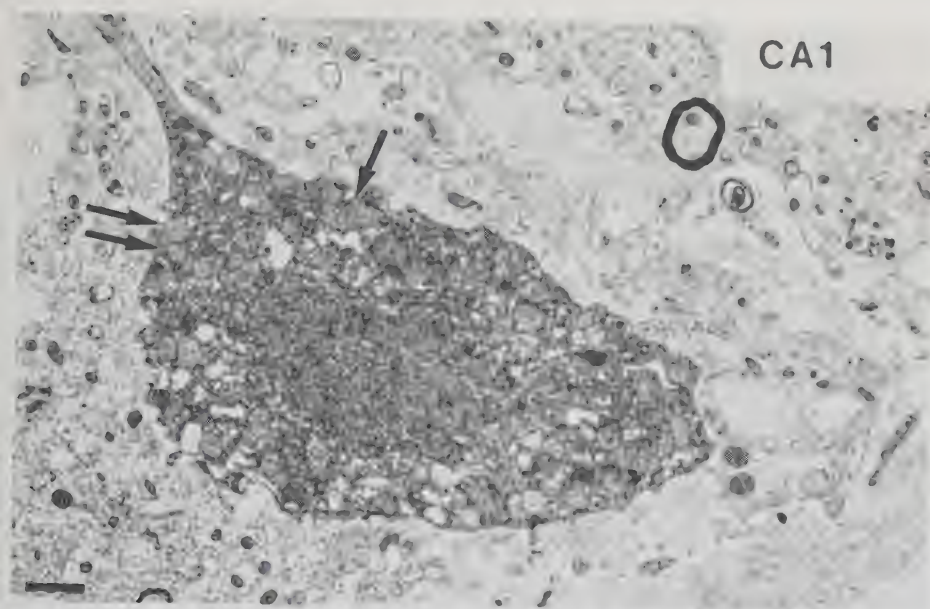
CA 1



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Figure 5. Laterally placed CA1 neurons, 3 wks after 30 min isoelectricity. There is a generalized increase in electron density of the cells, and nuclear changes exceed cytoplasmic changes. The nuclei are amorphous, and completely devoid of any chromatin pattern. The upper neuron is remarkably well preserved. There is mild dilatation of the endoplasmic reticulum, but mitochondria contain discernible cristae and do not contain flocculent densities (arrows). An adjacent neuron at a slightly more advanced stage of cell breakdown (lower) shows greater dilatation of the endoplasmic reticulum, but flocculent densities are notably still absent from mitochondria. Cytorrhesis is beginning at the lower right. Bar = 2 μ m.

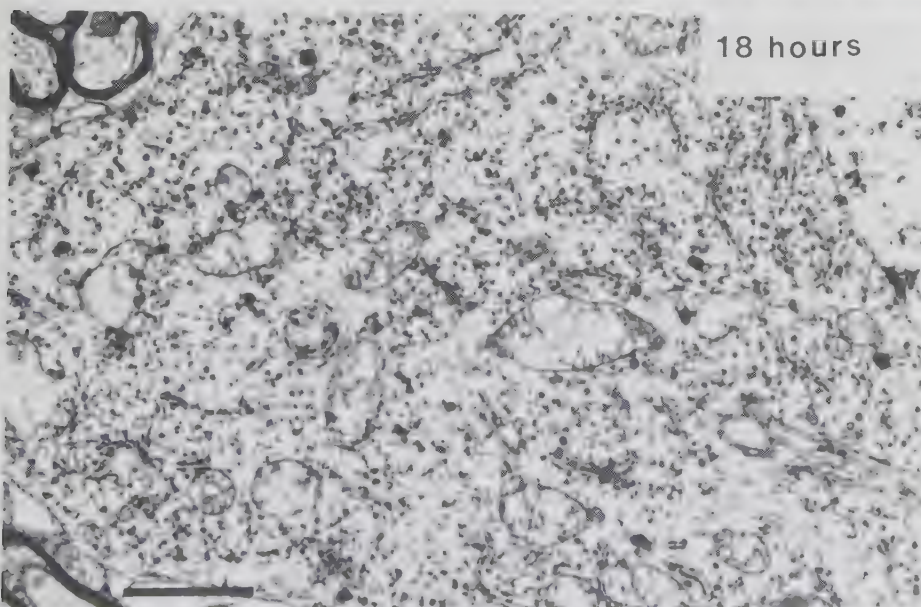
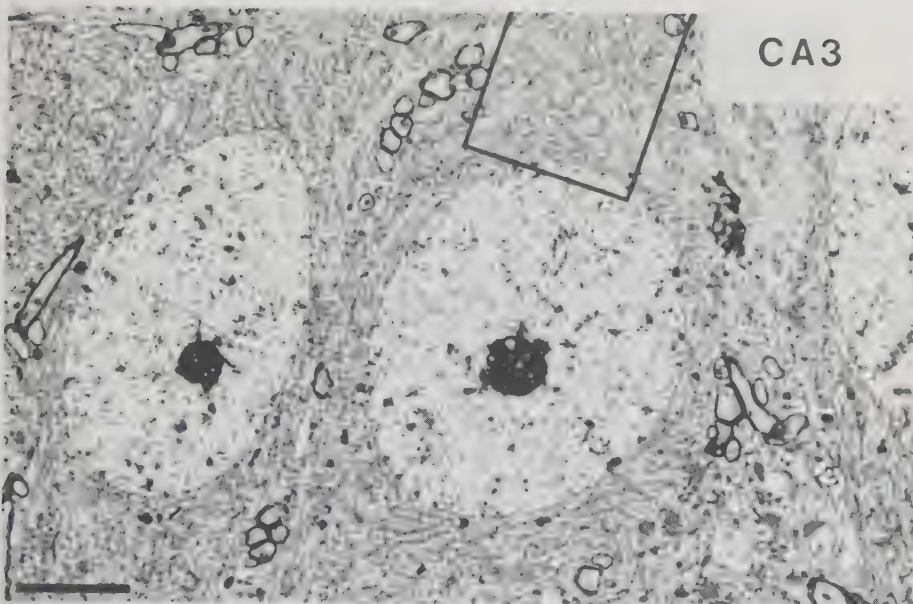
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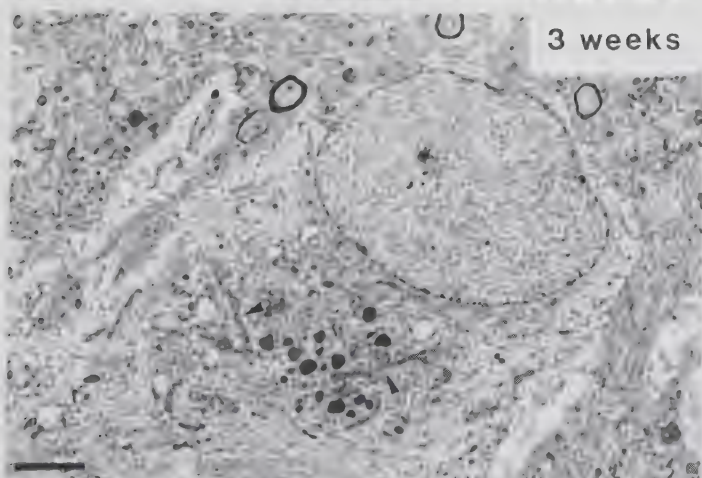
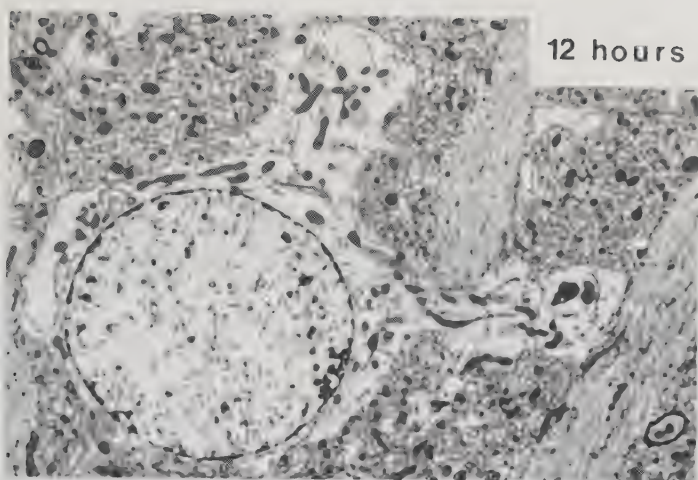
Figure 6. A row of CA3 pyramidal neurons, 18 h after 30 min of isoelectricity, show apparently little change at low magnification, although mossy fiber axons undergoing dark degeneration are seen (upper). A higher magnification (lower) of the area of neuronal cytoplasm indicated in boxes, however, reveals grossly disturbed mitochondrial architecture, with fractured cristae and varying degrees of swelling. Bars = 4 μm (upper), and 1 μm (lower).

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Figure 7. Sequence of astrocytic changes seen in both the neocortex and CA1 zone of hippocampus. After 12 h recovery following 30 min isoelectricity, the astrocytes show numerous elongated mitochondria in an expanded watery cytoplasm. At one week recovery, astrocytes with no intervening cell membranes are seen. There is cytoplasmic hypertrophy with fibril accumulation, and increase in cellular organelles. Numerous dilated cisternae of Golgi apparatus are seen. Effete mitochondria are numerous (arrowheads). After 3 wks recovery, residual bodies and lysosomes are now more numerous in the central cytoplasm of this hypertrophic astrocyte. Glial fibrils abound in the peripheral cytoplasm. Bar = 4 μ m.



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Figure 8. After one h recovery, hippocampal and neocortical capillaries demonstrate numerous microvilli which often branch, and many of which project a considerable distance into the lumen. Endothelial pits (arrows) and vesicles (arrowheads), are prominent. Bar = 1 μ m.

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By one week, densely fibrillated astrocytes were seen without intervening cell membranes between nuclei (Fig. 7, middle). Numerous degenerating mitochondria were seen with a homogeneous interior, containing only remnants of cristae. Multiple Golgi complexes with irregular dilatation of the cisternae were seen (Fig. 7, middle).

At three weeks, there were numerous effete mitochondria and residual bodies in an enlarged cytoplasm. Endoplasmic reticulum and glial fibrils were prominent (Fig. 7, lower).

Blood vessels

The capillaries of the CA1 zone showed changes resembling those seen in the cerebral cortex (4), consisting of prominent endothelial pits and vesicles in the cytoplasm. Numerous long and branching microvilli were seen, often projecting a considerable distance into the lumen of endothelial cells (Fig. 8). This appearance persisted for the first few days after the insult, and was more prominent after 60 min isoelectricity than after 30 min isoelectricity.

DISCUSSION

The present findings confirm those of the previous paper that acidophilic neurons evolve through time via a transformation from dark neurons (4). This evolution, as seen most clearly in the medial CA1 and subicular neurons, represents the transformation of a potentially reversible cellular injury into an irreversible one. Discussion of this topic can be found in the previous paper (4).

We will discuss the results presented above in relation to the questions outlined in the introduction.

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Delayed Neuronal Death

In the gerbil, it has now been well established that following short periods of ischemia, from three to five min, neuronal death in the CA1 pyramidal cells occurs following an interval where morphologic abnormalities are minimal to absent (21,22,23,24,35,36). The neuronal cytolysis occurs abruptly after this morphologic "free interval", with rapid removal of neuronal perikarya from the neuropil (21,22).

The disappearance of CA1 neuronal perikarya from the neuropil in lateral locations near the border with CA3, was found to be delayed in the present study. Thus, cytolysis occurred after one to six weeks in these locations, and the outlines of the cell membranes and cellular organelles were still visible in many neurons after three weeks. However, we found no evidence of a morphologic free interval, in the sense that neurons with configurational or tinctorial alterations appeared during recovery. Furthermore, the electron microscopic appearance of these neurons reported here in hypoglycemia differs considerably from that reported in ischemia (22,23). In contrast to the proliferation of rough endoplasmic reticulum seen in ischemia, the present results in hypoglycemia showed the greatest change in the nucleus, consisting of amorphous gray nucleoplasm with total loss of discernable chromatin, accompanied by a uniform increase in the electron density of nucleus and cytoplasm. This distinct appearance, with the greatest change in the nucleus, may indicate a nuclear rather than a cytoplasmic death in these cells.

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Hence, although neuronal cytolysis occurred after a delay in the lattermost CA1 pyramidal neurons, both the time of occurrence and the electron microscopic appearance of the cell damage were sufficiently different to distinguish these two forms of delayed neuronal death.

Temporal Evolution of Neuronal Damage

In ischemia, the hypothesis has been advanced that the rate of evolution of neuronal damage is proportional to the severity of the insult (17). Subsequent results obtained thus far have borne out the validity of this hypothesis in ischemia in the CA1 sector of the gerbil (21,22). In hypoglycemia in the rat, hippocampal damage in the CA1 pyramidal cell band is more severe medially, marked by denser selective neuronal necrosis in the subiculum and adjacent CA1. There is a lessening gradient of neuronal necrosis toward the border with CA3. The present model of hypoglycemia thus offered a situation which could be exploited to test this hypothesis in this same neuroanatomical area in the rat in hypoglycemia. This variation in the density of neuronal damage and in the time course of neuronal death along the hippocampal pyramidal ribbon cannot be explained by the neurochemical organization of this structure (38).

The present results have demonstrated that indeed, in the medial subiculum, especially the portion exposed to the subarachnoid space, evolution of dark neurons to acidophilic neurons occurs extremely rapidly, within two hours. The electron microscopic appearance of the neurons at two hours fulfils previously established criteria for neuronal death, consisting of flocculent densities in the mitochondria (14,18,19,37), rupture of the nuclear

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and cytoplasmic membranes, and a characteristic coarse stippling of the nuclear chromatin (4). This electron microscopic appearance corresponds to the acidophilic neurons of light microscopy (4).

The predominantly nuclear changes outlined above, in the neurons undergoing delayed neuronal cytolysis, contrast with the preponderance of cytoplasmic abnormalities seen in the classic acidophilic neuronal necrosis seen more medially. Hence, the possibility is raised that the lethal injury may occur in the nucleus in the delayed form of neuronal death, and in the cytoplasm or cell membrane in classic acidophilic neuronal necrosis. Cell membranes were notably intact even at three weeks in the lateral CA1 neurons undergoing delayed degeneration.

The examination of the portion of the subpial subiculum was undertaken specifically with the previously outlined hypothesis in mind of a CSF borne neurotoxin causing cell damage in hypoglycemia accompanied by isoelectricity (3). The segmentally swollen dendrites, containing swollen mitochondria with disrupted cristae were absent in control material. These dendritic abnormalities appeared in the stratum radiatum already after 10 min cerebral isoelectricity. The mitochondria seen resembled those seen in the perikarya of cerebral cortical and hippocampal pyramidal neurons during and after hypoglycemic isoelectricity, and represent, as discussed in the previous article, a non-specific response to injury (4).

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Reactive Changes in CA3

Here, the changes observed after 30 min isoelectricity can be safely regarded as reversible, since neuronal necrosis is virtually absent in the present model after this time interval (2). Even after 60 min, only CA3 pyramidal cells near the ventricles in animals with hydrocephalus are damaged (3).

In confirmation of this, no neuronal necrosis was seen in these cells in the present study. However, mitochondrial injury was present in the cytoplasm of numerous CA3 cells, even after only 30 min isoelectricity. The absence of neuronal necrosis in this region of the brain after this dose of insult, and the failure of these changes to evolve into neuronal necrosis in the present study, indicate the reversibility of this degree of mitochondrial injury in these neurons. The non-lethal nature of such mitochondrial neuronal damage has been previously established for CA3 neurons in ischemia (29), and in neuronal types other than CA3 pyramidal cells in both ischemia (28) and hypoglycemia (4).

Astrocytes

The sequence of alterations in the astrocytes of CA1 was identical to those previously alluded to in the superficial cerebral cortex (4). A similar astrocytic response has been reported in ischemia (27). The proliferation of astrocytic mitochondria after several hours suggests a reactive response of the astrocyte requiring increased energy production. It was not due to neuronal necrosis, since such astrocytes appeared in areas devoid of dead or dying neurons.

By one week numerous degenerate mitochondria were seen, with a homogeneous interior lacking the normal cristae, or showing only remnants of cristae. These effete mitochondria were interpreted to

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be derived from the numerous mitochondria seen after several hours recovery, and could be seen evolving from them at intermediate time intervals. The Golgi apparatus became more prominent and numerous than usual, accompanied by synthesis of cytoplasmic fibrils.

By three weeks, there were considerable aggregations of residual bodies in the cytoplasm, with some effete mitochondria and intermediate forms remaining.

In view of the evolution of these events in the astrocytic cytoplasm, this sequence is interpreted as a response to demands for increased energy production in the astrocyte, associated with a reactive process causing increased mitochondrial turnover. Cultured astrocytes have been shown to degrade ingested mitochondria into cytoplasmic residual bodies by two wks (10). Indeed, the cytoplasmic appearance of the astrocytes at 3 wks is quite similar to that pictured in reactive neuronal cytoplasm of CA3 neurons, 3 wks following ischemia (9).

Blood Vessels

The endothelial response in the CA1 zone was similar to that seen in the cerebral cortex in this model (4), with the formation of endothelial pits, vesicles, and branching microvilli. Such changes were confined to the capillary portion of the microcirculation, and were not seen in arterioles or venules.

Cerebral capillary endothelial cells normally have few vesicles or microvilli (12,25,26,32), these structures perhaps indicating increased vascular permeability (25). Cerebral endothelial microvilli have also been reported after ischemia (12,16), in hypertension (15), and in traumatic brain injury (30), and hence must be considered a nonspecific response to injury. Still, the

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results demonstrate that hypoglycemia accompanied by isoelectricity leads to alterations of glial cells and capillaries as well as neurons.

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THE TEMPORAL EVOLUTION OF HYPOGLYCEMIC BRAIN DAMAGE.

III. LIGHT AND ELECTRON MICROSCOPIC FINDINGS IN THE RAT CAUDOPUTAMEN

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ABSTRACT

The caudate nucleus and putamen belong to the selectively vulnerable brain regions which incur neuronal damage in clinical and experimental settings of both hypoglycemia and ischemia. We have previously documented the density and distribution of the hypoglycemic damage in rat caudoputamen, but the evolution of the injury, i.e. the sequence of structural changes, has not been assessed. Therefore, in the present study we analyze the light and electron microscopic alterations in the caudoputamen of rats exposed to standardized, pure insults of severe hypoglycemia with isoelectric EEG for 10-60 min, or in rats which, following insults of 30 or 60 min, were allowed to recover for periods from 5 min to 6 months.

The hypoglycemic insult produced severe nerve cell injury in the dorsolateral caudoputamen. Immediately after the insult abnormal light neurons with clearing of the peripheral cytoplasm were present. These cells disappeared early in the recovery period, as they do in the cerebral cortex. Dark neurons were also present, but unlike those in the cerebral cortex they did not appear until recovery was instituted. Their number increased for a couple of hours and they became acidophilic within 4-6 h. At this stage electron microscopy revealed severe clumping of the nuclear chromatin and cytoplasm as well as incipient fragmentation of cell membranes, all these changes indicating an irreversible injury. Within 24 h flocculent densities appeared in the mitochondria and by day 2-3 of recovery the great majority of the medium sized neurons had undergone karyorrhexis and cytorrhexis, their remnants being subsequently removed by macrophages. After some weeks only large and a few medium sized neurons remained amidst reactive astrocytes and numerous macrophages.

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The delay in the appearance of dark, lethally injured medium-sized neurons until the recovery was instituted suggests an effect that does not become apparent until the substrate supply and energy production are restored. Furthermore, it points out again the selectivity of the hypoglycemic nerve cell injury with respect to the type (metabolic characteristics?) and topographic location of the neurons.

INTRODUCTION

The caudate nucleus and putamen (caudoputamen) belong to the brain structures prone to develop ischemic damage. This damage is pronounced in ischemia due to middle cerebral artery occlusion which obstructs flow to the nuclei (for further details see ref.13). Damage is inflicted upon the caudoputamen also in severe forebrain ischemia, with characteristic localization to small and medium sized neurons in the dorsolateral crescent of the nucleus (26). These neurons, therefore, belong to the selectively vulnerable brain cells, and at least some of them have been suggested to be GABAergic (14). However, such selectively vulnerable cells in various regions of the brain differ in several respects, e.g. in the rate at which they develop signs of irreversible injury following an ischemic insult. Thus, whereas CA1 pyramidal neurons of the hippocampus develop such signs first after a free interval of 2-3 days (15,21,22,27) neurons in the caudoputamen succumb much quicker without a demonstrable free interval.

In humans dying after episodes of severe hypoglycemia the caudate nucleus and the putamen are also frequently damaged (12,19,23). Corresponding hypoglycemic damage has also been demonstrated in subhuman primates after long term survival by Myers and Kahn (24),

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whereas less consistent injury was reported by Brierley et al. (10) after short recovery periods. In all cases it remained unsettled, whether secondary ischemia could have contributed to the lesions. For several years our laboratories have been investigating the neuropathological features of pure hypoglycemic insults delivered to artificially ventilated rats with adequate blood pressure and arterial oxygenation. Earlier studies helped to characterize light and electron microscopic features of hypoglycemic brain injury during the insult or in the immediate post-insult period (3,4,18). In these studies, though, the caudoputamen was not examined. With the development of a long-term recovery model it became feasible to study both the density and the distribution of the final hypoglycemic brain damage (6,7). These studies showed that pure hypoglycemic insults caused extensive damage to the caudoputamen with a distribution which was similar but not identical to that reported following ischemia (26). Thus, with mild insults neuronal damage was localized to the lateral parts of the nucleus, whereas more severe insults caused damage to its superior parts (7).

None of the studies quoted gave information on structural alterations in the caudoputamen during the insult, nor on their temporal sequence. In order to assess the evolution of hypoglycemic cell injury in key brain structures, we have started a series of investigations to evaluate the light and electron microscopic alterations during the insults, as well as following short and long recovery periods. Two previous articles in this series have been devoted to the neocortex and the hippocampus (8,9), while the present one concerns the caudoputamen. The following questions are addressed. Firstly, do nerve cells of different type react differently to the hypoglycemic insult? Secondly, is the sequence of alterations similar to that observed in the neocortex and

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hippocampus, e.g. do dark neurons appear during the insult and become transformed to acidophilic ones during recovery? Thirdly, is the rate of cell death in the caudoputamen different from that in other areas examined? Finally, what is the fate of the irreversibly injured neurons and what is the tissue reaction to the injury?

To answer the questions posed the sequence of the light and electron microscopic alterations was examined in rats subjected to insulin-induced hypoglycemia with isoelectric EEG lasting for 10, 20, 30 or 60 min. The animals were sacrificed either immediately after the insult or, following insults of 30 or 60 min, after a recovery period ranging from 5 min to 6 months.

MATERIAL AND METHODS

Animal Model

A previous publication describes in detail the model used (6). The first publication in this series (8) gives an account of the procedures used in the present experiments, and the material presented here is derived from the same animals.

Light and Electron Microscopic Procedure

Perfusion fixation and histologic processing were performed as reported in the first article of this series (8). Brains were sectioned coronally into 1.8 mm thick slices, and one half of the brain was used for light microscopy (LM) in a soft plastic polymer embedding medium (EFL-67, Serva Feinbiochemica, Heidelberg, West Germany). Sections were cut at 1.5 μ m and were double stained with acid fuchsin and cresyl violet. Toluidine blue stained semithin epon sections (see below) were also used for LM.

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The opposite hemisphere was used for electron microscopy. Samples from the caudate nucleus at the level of the septal nuclei were processed to be embedded in Epon 812. Semithin sections stained with toluidine blue were used for the LM evaluation and for the selection of the areas to be thin-sectioned for electron microscopy (EM). The thin sections were double stained with uranyl acetate and lead citrate and examined with a JEOL JEM 100 C electron microscope.

RESULTS

In the control animals the structure of the neurons was such as is generally considered normal. Some irregularity of the myelin sheaths was observed, since glutaraldehyde fixation does not give faultless preservation of the myelin structure in tightly packed bundles.

Hypoglycemia Without Recovery

Immediately after 10 min of isoelectric hypoglycemia neither the LM nor the EM structure differed significantly from the controls. Only the mitochondria of most neurons appeared normal or were slightly compact. After 20, 30 or 60 min of hypoglycemia the general structure of the tissue by LM was fairly compact, i.e. no major sponginess was observed. Many neurons (Fig 1) showed clearing of the peripheral cytoplasm, i.e. so-called type 2 injury (3,18). It was most obvious in larger neurons and with longer duration of the hypoglycemia; yet it did not become as prominent as in the overlying cortex.

The cytoplasm of many astrocytes appeared pale and enlarged and the perinuclear part as well as many processes stood out much more clearly than normally (Fig 1). They began to resemble smaller

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neurons with the peripheral clearing, which sometimes made the light microscopic distinction between these cells difficult. In many astrocytes after longer durations of hypoglycemia the enlarged watery cytoplasm also contained vacuoles and membranous whorls. Their nuclear chromatin was typically homogeneous in the slightly enlarged nuclei (Fig 1B). Evidently the small vacuoles which appeared in the neuropil were due to this astrocytic edema.

The oligodendroglial cells did not show prominent changes, only their nuclei appeared somewhat lighter than normal. Similarly, myelin did not differ significantly from that in the controls (see Fig 1A). The blood vessels appeared intact, but slight perivascular edema was observed around some larger vessels.

By EM the "peripheral clearing" in neurons was verified to be due to gathering of the organelles in the perinuclear area (Fig 7). Cisternae of the rough endoplasmic reticulum (RER) were narrow and elongated, and mitochondria were most often compact with darkly staining inner matrix (Fig 7). However, there were some neurons (without obvious clustering of the organelles) in which the mitochondria showed mild to moderate swelling. The nuclear chromatin of the neurons was evenly distributed (Figs 1B and 7).

Hypoglycemia Followed by Recovery

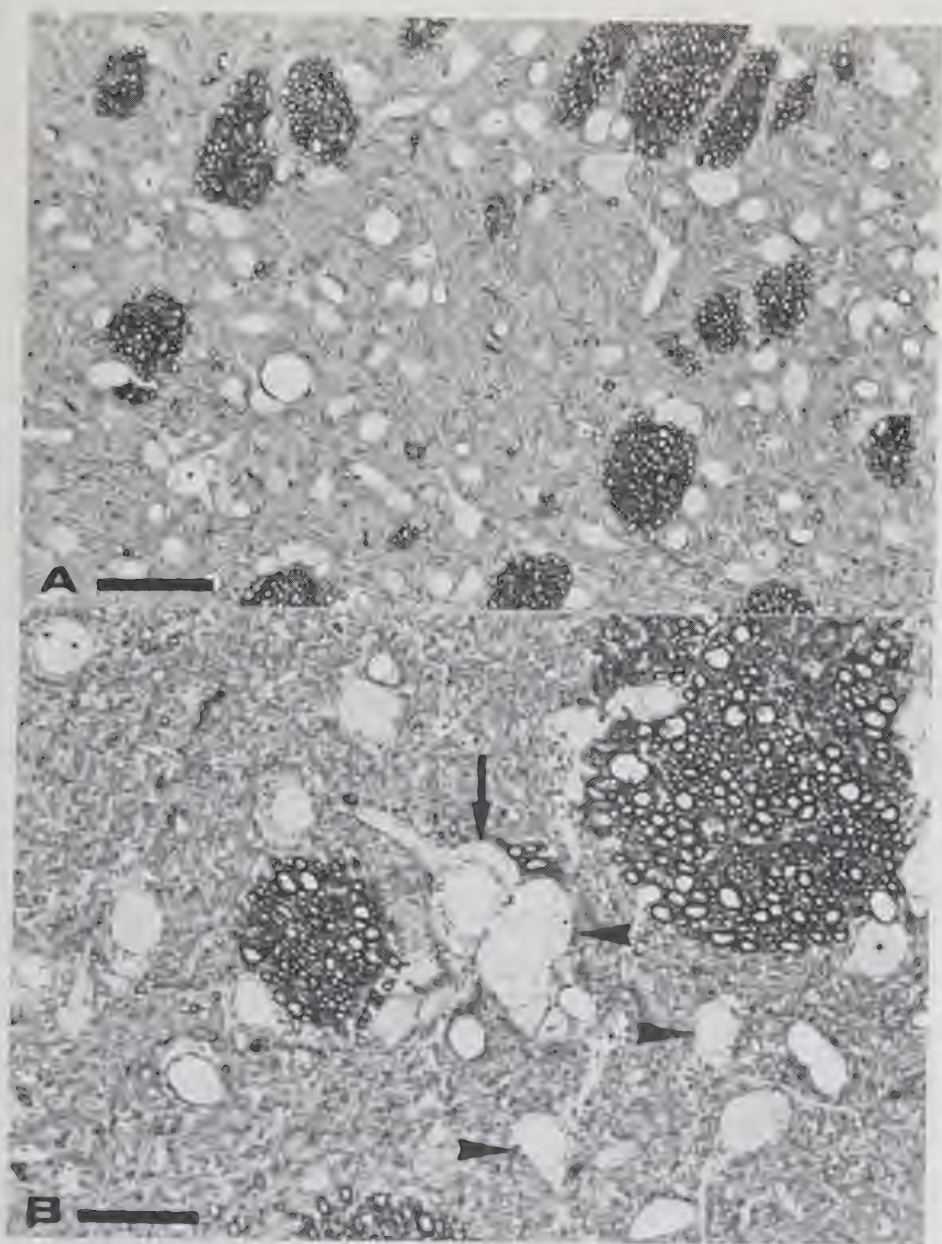
After the restoration of normoglycemia structural changes appeared within minutes. Though most neurons appeared intact (Figs 2A, 8), dark, condensed neurons were observed already after 5 min of recovery following 60 min of isoelectric hypoglycemia (Fig 2) and they became more striking and numerous with longer restitution (Fig 3). These dark cells were located mainly in the dorsolateral parts of the caudoputamen, the medial parts of the nucleus being

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Figure 1A. Overview of caudato-putamen after 60 min of isoelectric hypoglycemia without restitution. Only minor changes can be observed. Neurons show clearing of their peripheral cytoplasm, but no dark neurons are seen. Astrocytes are slightly edematous but in general the tissue appears compact. Bar = 50 μ m.

Figure 1B. Higher magnification to demonstrate the clearing of peripheral cytoplasm in a larger neuron (arrow). The astrocytes (arrow heads) have enlarged pale cytoplasm, which evidently contributes to the minimal sponginess of the neuropil. Astrocytic cytoplasm may contain vacuoles and membranous whorls. The nuclei of all cells have evenly distributed chromatin. Bar = 20 μ m.

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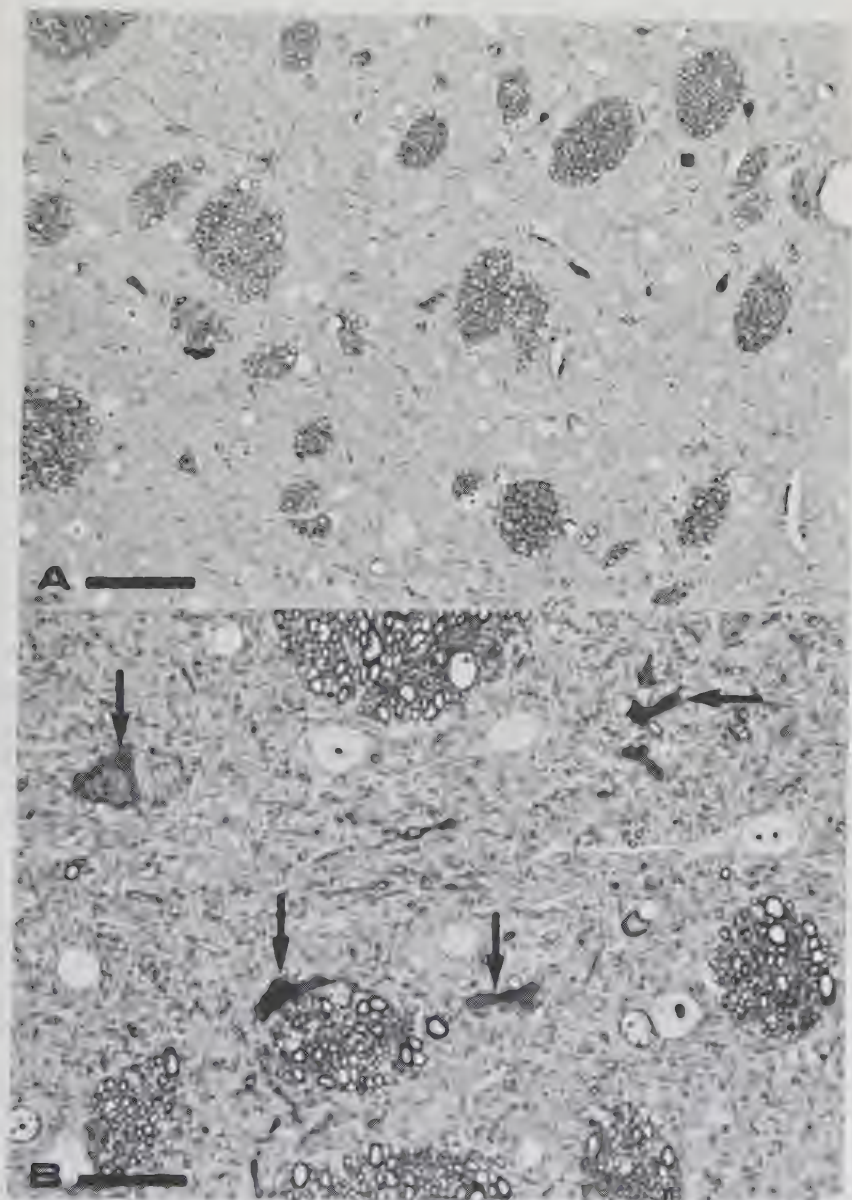


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Figure 2A. Already after 5 min of restitution following 60 min of hypoglycemia dark, condensed neurons appear in caudoputamen, mainly in the rostral dorsolateral part. In general, the tissue appears compact, though some of the dark neurons are surrounded by small vacuoles, and astrocytes are slightly edematous. Bar = 50 μ m.

Figure 2B. A higher magnification from the same animal as in Fig 2A shows that the condensation of the neurons (arrows) is usually not preceded by edema. The neuropil has remained remarkably compact. Next to the dark neurons there are several normal appearing ones. Bar = 20 μ m.

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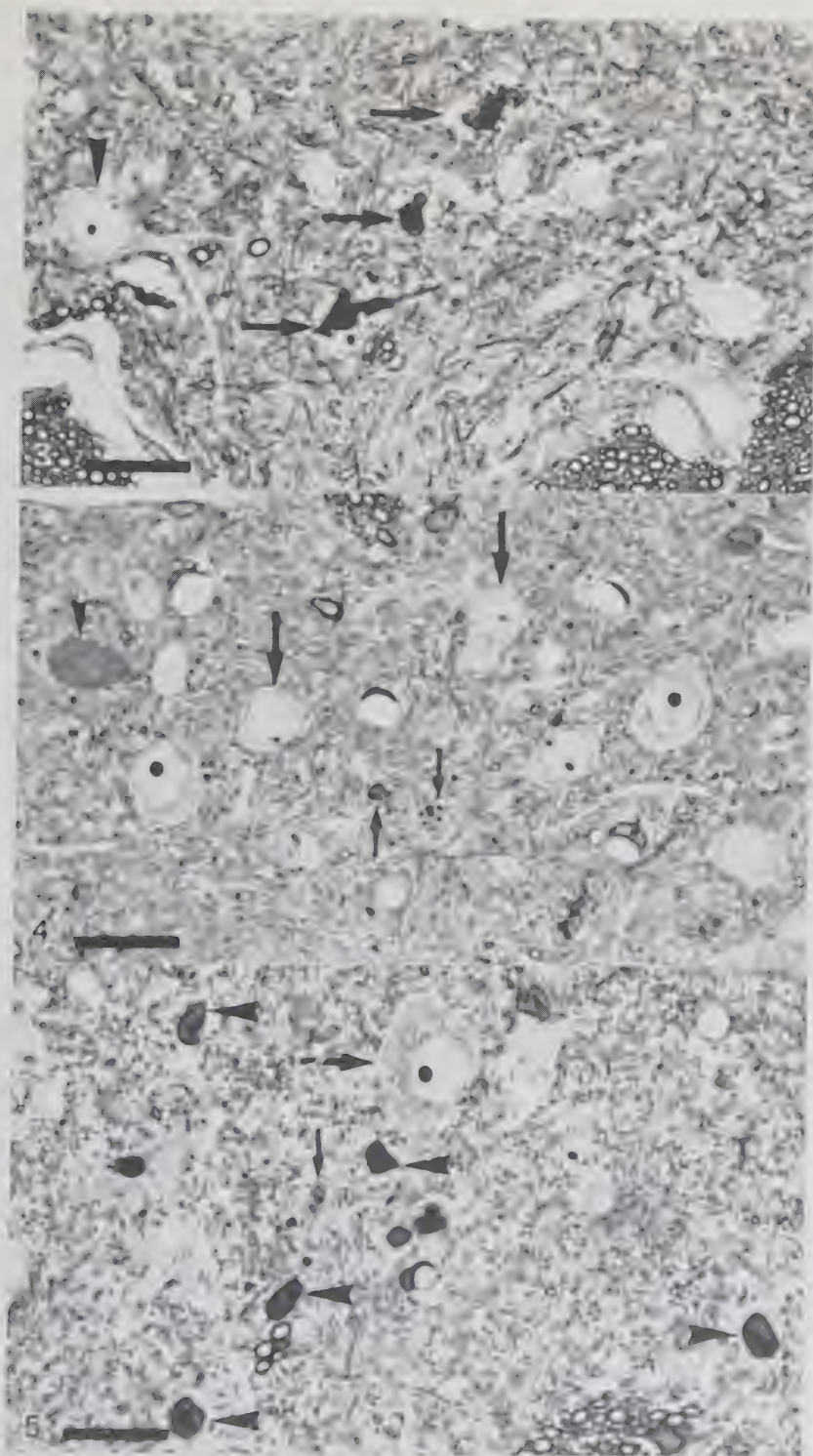


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Figure 3. After 3 h restitution following a 30 min hypoglycemic insult the injured neurons (arrows) have become very dark, and their outlines have become more irregular indicating incipient fragmentation of the cytoplasm and nucleus. A few tiny cytoplasmic vacuoles are seen and there is definite perineuronal vacuolation. The larger neuron (arrow head) appears fairly intact. The astrocytes have a watery cytoplasm and the neuropil shows moderate sponginess. The bundles of myelinated fibers are not noticeably affected. Bar = 20 μ m.

Figure 4. By two days after 30 min of hypoglycemia most of the dark neurons which appeared earlier have undergone fragmentation (karyo- and cytorrhesis) and are hardly discernible (smaller arrows). A moderately dark neuron (arrow head) suggests that a few neurons may become injured later during the restitution. Two larger neurons appear intact. The astrocytes (larger arrows) often have prominent nucleoli and abundant cytoplasm, thus sometimes closely resembling neurons. The neuropil is slightly spongy. Bar = 20 μ m.

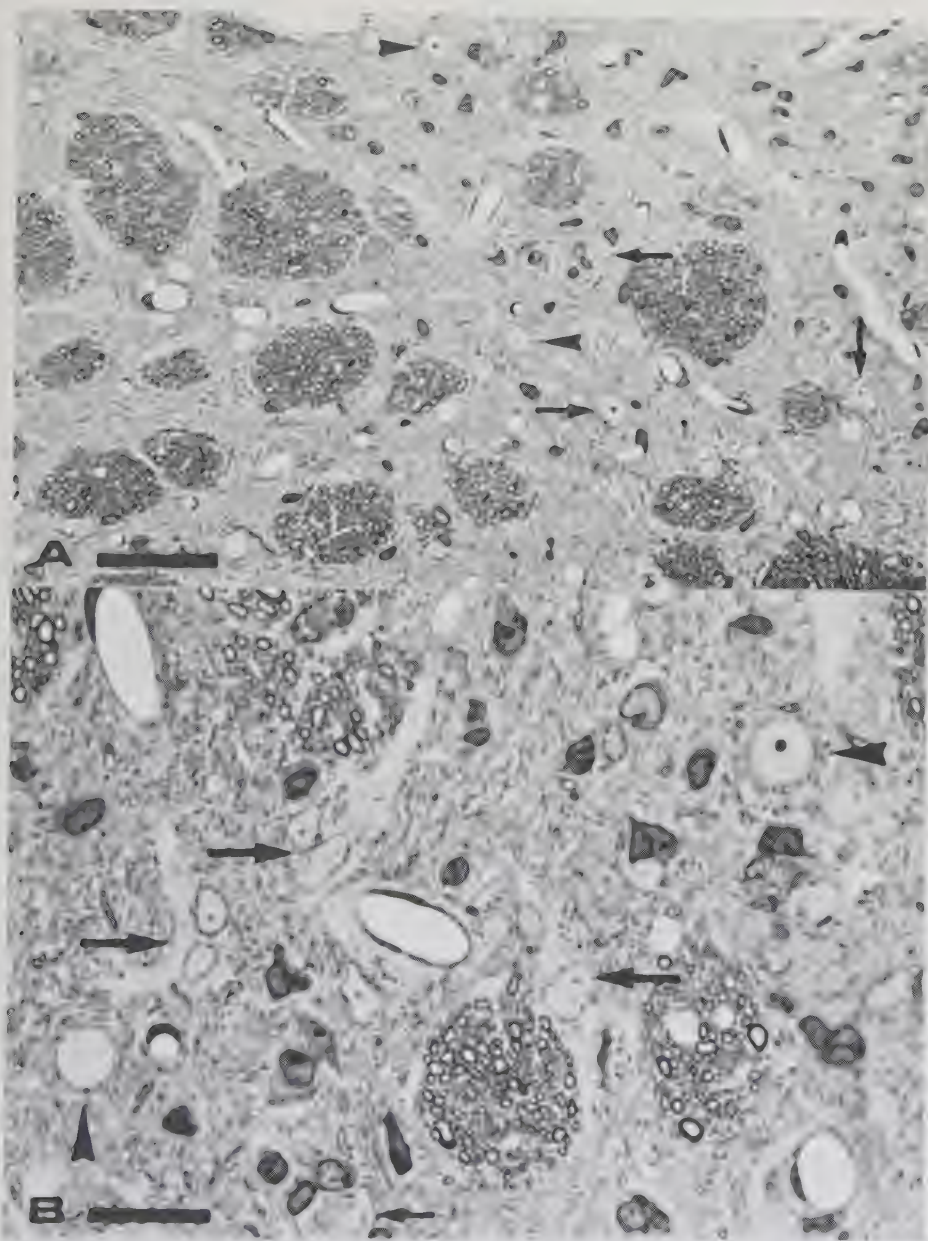
Figure 5. After three days of restitution following 30 min of hypoglycemia the remaining fragments of the karyo- and cytorrhectic cells are hardly discernible (small arrow). The dark compact cells (arrow heads) are macrophages which appear in the tissue at day 1-2 of restitution. The astrocytes are prominent. The neuropil shows generalized sponginess. Typically, the large neuron (large arrow) has been preserved. Bar = 20 μ m.



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Figure 6. After 6 weeks restitution following 30 min hypoglycemia, some medium-sized neurons (small arrows) are visible in the less severely affected area (A, lower left). In the more severely affected part (upper right) most medium-sized have disappeared, but two neurons (arrow heads) are visible among the numerous dark-staining macrophages. Reactive astrocytes (large arrows) are prominent. The bundles of the myelinated fibers appear virtually normal and the neuropil no longer appears spongy. These changes are seen in greater detail below in B. Bar = 50 μ m

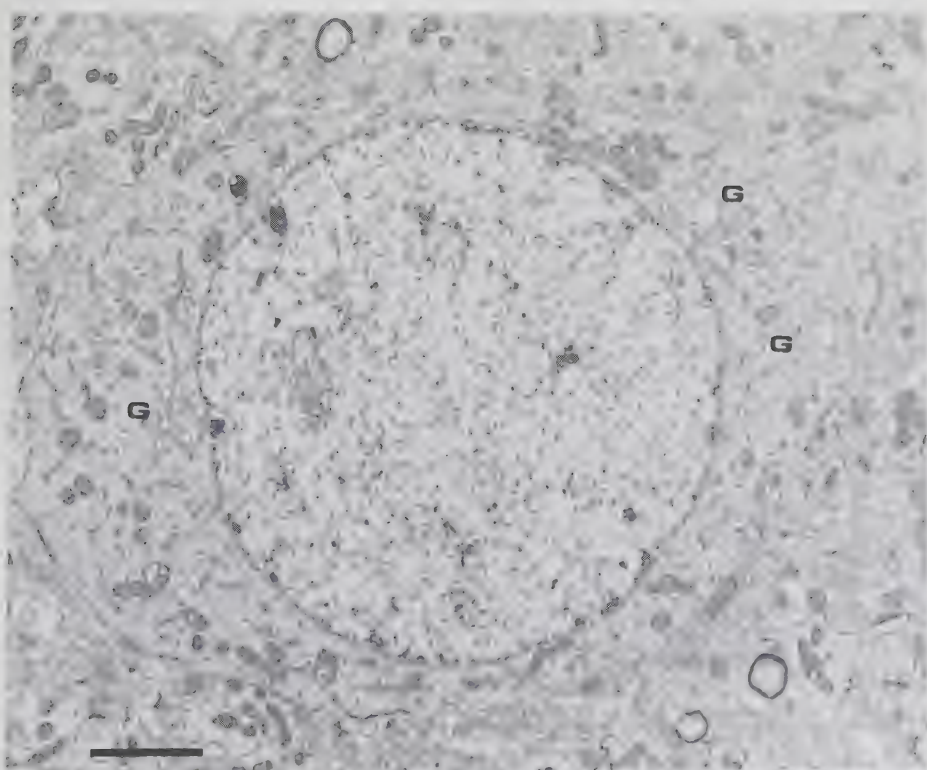
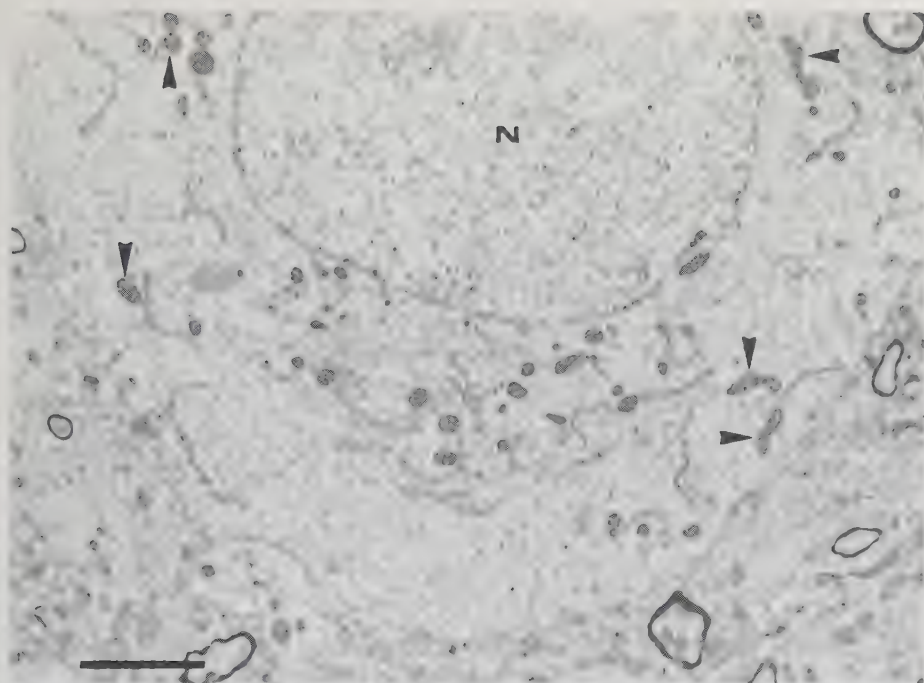
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Figure 7. Immediately after the hypoglycemic insult (in this picture 30 min of isoelectricity) the cytoplasmic organelles are clustered around the nucleus (N) resulting in "clearing of the peripheral cytoplasm" (cf. Figs. 1A and B). The ribosomes have lost their compact structure and rosette formation. RER cisternae have remained narrow. Mitochondria (some marked with arrow heads) are contracted with dark staining inner matrix. The nuclear chromatin is evenly distributed. Bar = 2 μ m.

Figure 8. After short periods of restitution (in this case 10 min) following 30 min of hypoglycemia most medium-sized neurons still showed fairly few changes: The mitochondria appear less contracted, Golgi cisternae (G) are somewhat dilated and ribosomes are pinpoint, with partial loss of rosette formation. Nuclear chromatin remains homogeneous. Bar = 2 μ m.

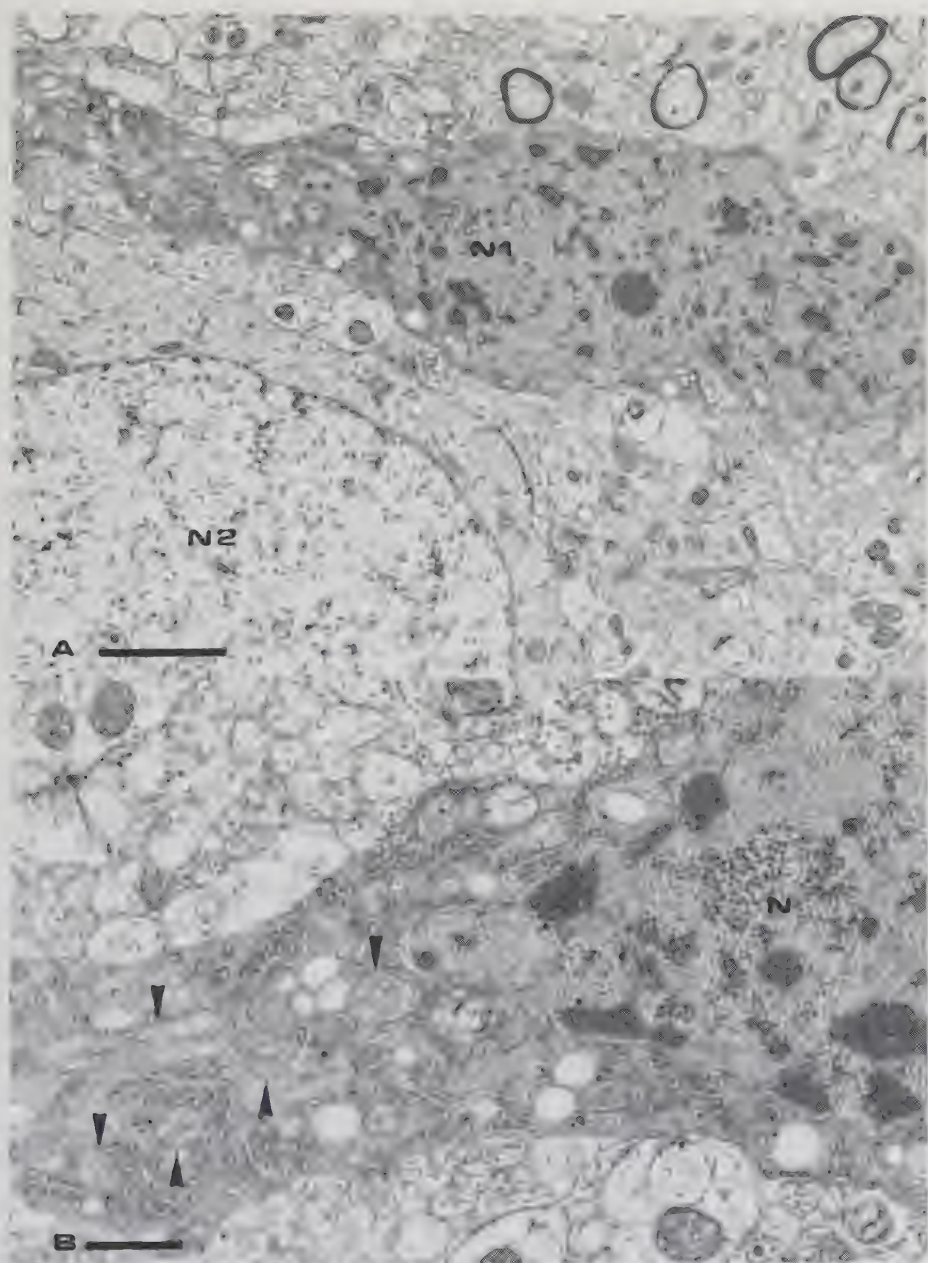


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Figure 9A. Already after 5-10 min of restitution dark condensed neurons of medium size (N1) appeared. Both the karyo- and cytoplasm are markedly electron dense. Some small vacuoles are visible in the cytoplasm. Remarkably, there is no prominent perineuronal edema. The second neuron (N2) corresponds to that in Fig 8. Bar = 2 μ m.

Figure 9B. A higher magnification of the dark neuron shows the well preserved mitochondria (arrow heads) in the condensed cytoplasm. Ribosomes are compact and tightly packed. There are a few tiny vacuoles the exact nature of which cannot be determined. Bar = 0.5 μ m.

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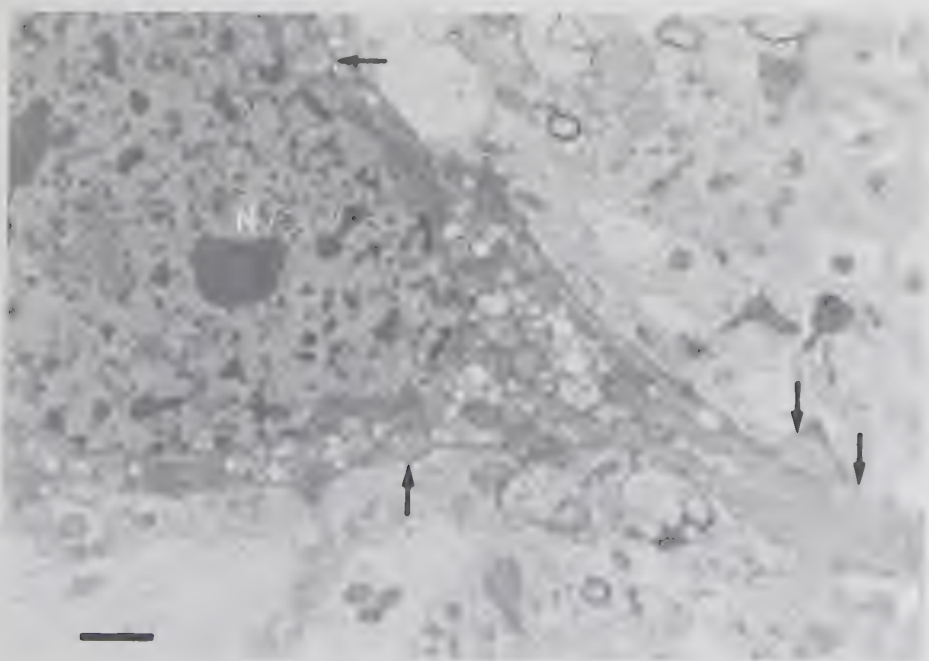
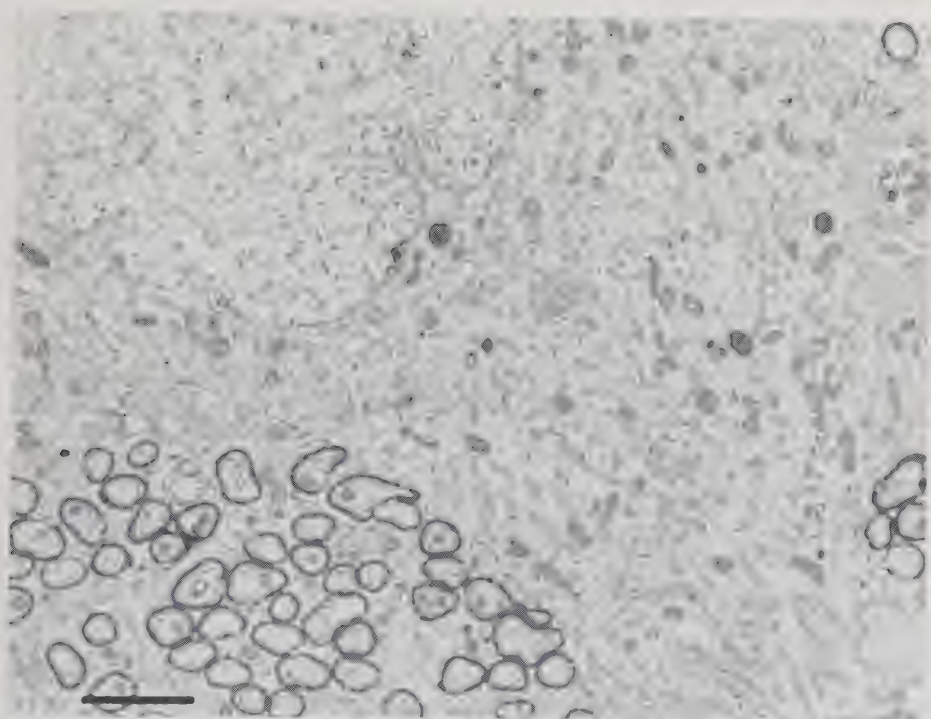


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Figure 10. A large neuron, which type seems to be very resistant, after 10 min of restitution following 30 min of hypoglycemia. The chromatin in the typically indented nucleus is evenly distributed, cytoplasmic organelles do not show any prominent change except for pinpoint diffusely arranged ribosomes. Bar = 2 μ m.

Figure 11. After a few hours of restitution (in this case 2 h following 60 min of hypoglycemia) mitochondria in the dark neurons show slight swelling and lighter staining of their inner matrix (arrows). Ribosomes and microtubules appear intact though tightly packed. The cluster of small vacuoles most likely represents dilated Golgi vesicles. The nuclear (N) chromatin is dark and coarsely clumped. In the neuropil both light, edematous and dark, condensed profiles are present. Bar = 1 μ m.

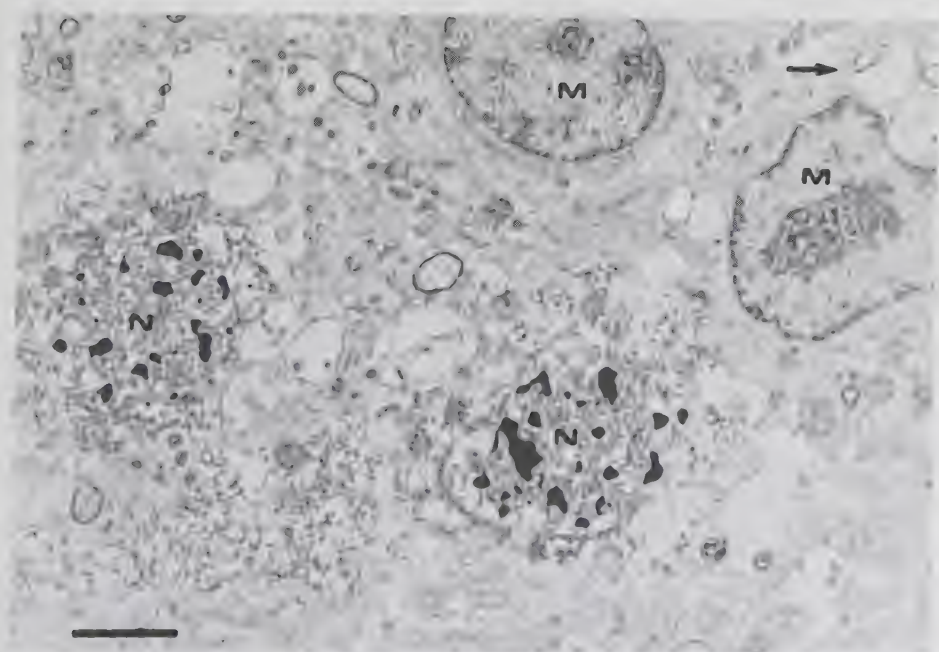
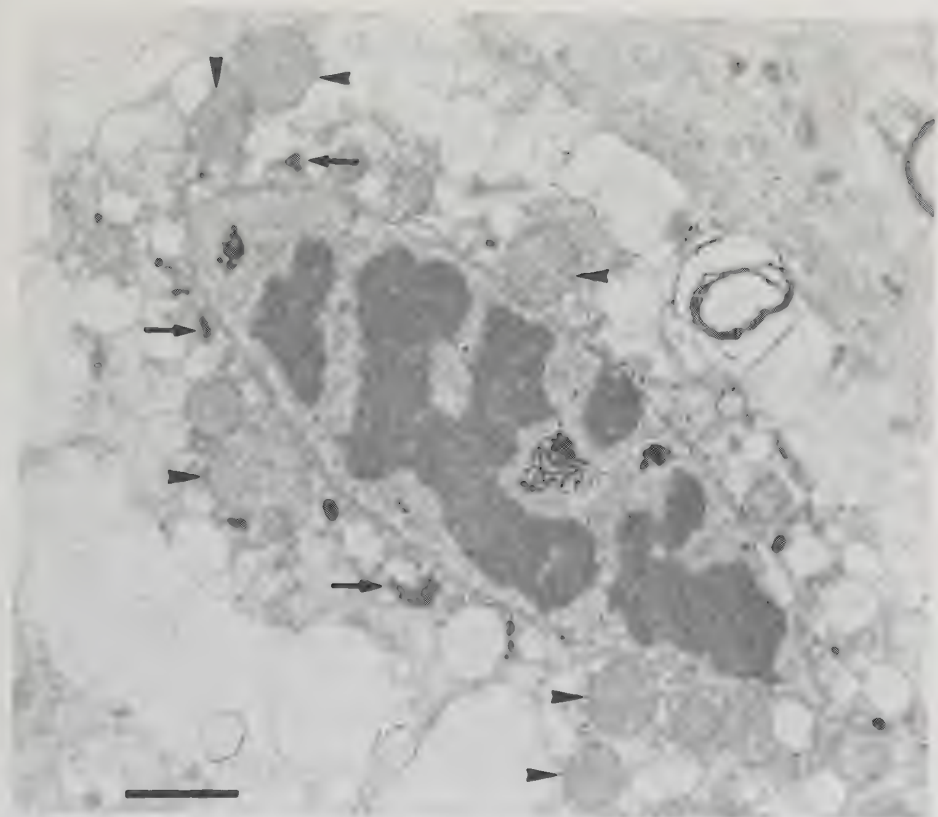
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Figure 12. One of the most severely affected neurons from the same animal as in Fig 11 is already disintegrating with fragmentation of the cytoplasm and possible leakage of dark material (arrows) from the nucleus, where the chromatin forms coarse clumps. Mitochondria (arrowheads) have remained remarkably compact, and definite intramitochondrial densities are not yet visible. Bar = 1 μ m.

Figure 13. After 2 days restitution following 30 min of hypoglycemia there are only remnants of two neurons (N). The nuclei have undergone karyorrhexis and are surrounded by necrotic cell debris. Macrophages have appeared to scavenge the necrotic material (arrow). Bar = 2 μ m.

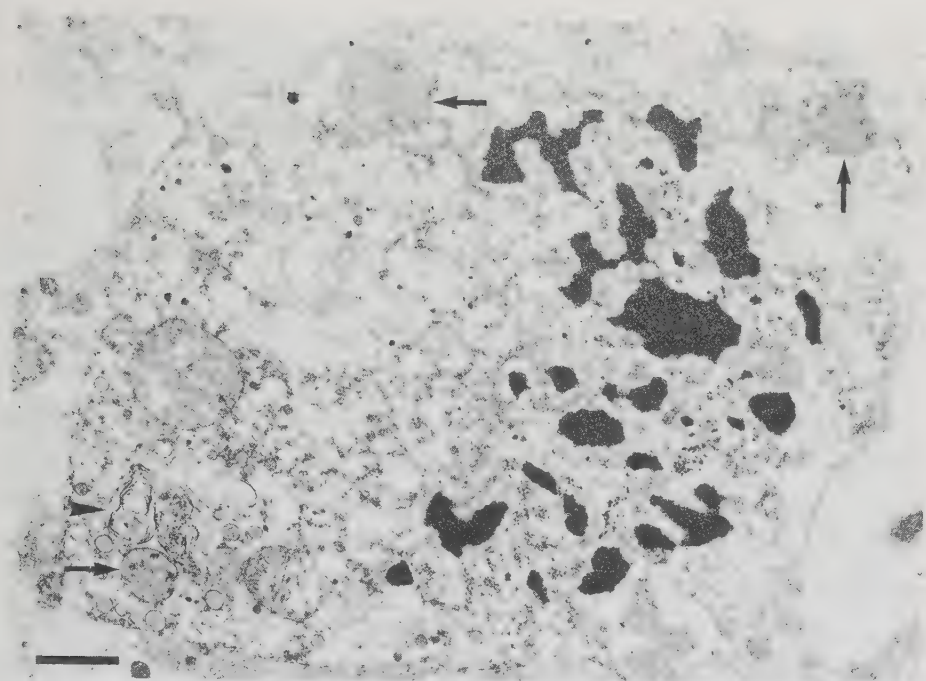


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Figure 14. A higher magnification of a similar neuron as the one in Fig 13, from a rat exposed to 30 min of hypoglycemia followed by 24 h restitution. The nuclear chromatin appears as dark clumps and the nuclear envelope has disappeared. The cytoplasm is composed of vesicular and granular material with a few membranous whorls (arrowheads). The surprisingly well preserved mitochondria (arrows) contain dark densities.
Bar = 1 μ m.

Figure 15. Six weeks after 30 min of hypoglycemia large reactive astrocytes (A) are common. Their cytoplasm is filled with glial filaments. Their organelles include many residual bodies, which are clustered on one side of the slightly grooved nuclei. A macrophage (M) with abundant cytoplasm is seen at the lower right.
Bar = 2 μ m.

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relatively spared. By LM, in both acid fuchsin/cresyl violet and toluidine blue stained sections the injured neurons appeared at first compact, angulated, dark blue stained. Remarkably they were not often surrounded by perineuronal vacuoles or spongy neuropil, i.e. the condensation was not preceded by edema (Figs 2, 9A). As the neuronal changes accentuated with prolonged restitution, the degree of edema around the neurons increased simultaneously (Fig 3). Microvacuoles in the cytoplasm of the condensed neurons were not seen until a few hours of restitution had elapsed (Fig 3). By 4-6 h (beginning at 2 h) the dark, condensed neurons stained red with acid fuchsin, i.e. became acidophilic.

By EM these neurons were correspondingly dark and condensed which made the distinction of the nucleus and cytoplasmic organelles difficult (Figs 9A, 9B and 11). The cytoplasmic clear microvacuoles seen in LM most often corresponded to dilated Golgi vesicles. Furthermore, many mitochondria showed increased electron lucency of their inner matrix, but extreme ballooning of mitochondria was very rare (Fig 11).

The astrocytic nuclei remained enlarged with evenly distributed chromatin. Frequently, they contained a small nucleolus (Fig 3). Their cytoplasm also remained clear and watery which was the obvious reason for the vacuolization of the neuropil as well as for the perineuronal and perivascular clear spaces (Figs 3, 11). Oligodendroglial cells and myelin did not show marked deviations from the controls (Fig 3).

After 4-6 h the condensed neurons began to break into fragments. This disintegration continued and by 1-2 days the damaged neurons appeared as karyorrhectic nuclei amidst dissolving cytoplasm (Fig 4). At the same time the degree of sponginess (i.e. tissue edema) increased considerably: there was perivascular, perineuronal as

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well as general neuropil vacuolation. By EM the fragmenting neuronal nuclei were composed of dark clumps and less dense granular material surrounded by remnants of cytoplasm with few recognizable organelles (Figs 12, 13, 14). However, although flocculent densities appeared in them within one day (Figs 13, 14), many mitochondria retained their structure surprisingly well in these disintegrating neurons. This process of destruction was obviously very rapid, since hardly any intermediate forms between the dark and disintegrating neurons were observed.

After 2-3 days in the severely damaged animals the majority of neurons were no longer discernible in toluidine blue stained sections, whereas red remnants could still be seen with the acid fuchsin stain. In EM sections scattered darkly stained neuronal fragments were present among the preserved cells (Fig 4). The remaining neurons often had watery cytoplasm and occasional swelling of mitochondria. Destruction of at least some neurons was seen in all animals exposed to 30 or 60 min of hypoglycemia. It appeared that most of the neurons destined to be destroyed were recruited during the first hours of restitution. However, occasional compact darkly stained neurons, similar to those present during the early restitution, were also detected at the later stages (Fig 4), suggesting delayed death of a few nerve cells.

Among the preserved neurons and the neuronal remnants, increased numbers of small cells with variable form and often elongated nuclei and coarse chromatin began to appear at day 1-2 of restitution (Fig 5). These newly arrived cells corresponded in EM to macrophages (i.e. microglial cells, Fig. 13): RER was abundant and their cytoplasm contained phagocytosed material. The astrocytes were still fairly prominent with enlarged electron lucent cytoplasm

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in which an increased number of mitochondria were present. Their nuclear chromatin retained the homogeneous distribution seen in the controls.

At the very late stages, i.e. some weeks to months after the insult the picture was characterized by a marked loss of neurons in a quite compact tissue with increased cellularity (Fig 6A). The predominant cells were small macrophages with fairly scanty, darkly stained cytoplasm and nucleus (Fig 6B). Scattered among these cells were some preserved larger neurons (Fig 10). Large, reactive astrocytes were also present in great numbers (Fig 6). They had a nucleus with homogeneous chromatin and a tiny nucleolus. The cytoplasm formed broad processes which were rich in glial filaments. The organelles, including many residual bodies, were usually gathered on one side of the slightly grooved nucleus (Fig 15).

DISCUSSION

Our discussion will be centered on the four questions posed in the introduction.

Types of Neurons Injured

The results demonstrate that hypoglycemia, like ischemia, spares some neurons, notably the large ones, even in areas (and animals) with dense neuronal necrosis. Thus, at least two factors determine the vulnerability of caudoputaminal neurons to hypoglycemic insults: the topographical location (e.g. lateral and/or superior parts) and the cell type. Although direct proof is lacking, it is tempting to conclude that at least part of the medium sized neurons affected are GABAergic (14).

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Evolution of Structural Alterations and Rate of Cell Death

The same basic types of neuronal alterations were encountered in the caudoputamen as in the cerebral and hippocampal cortex of rats subjected to severe hypoglycemic insult with isoelectric EEG (3,8,9,18). These comprised (1) angulated, condensed, darkly staining neurons with perineuronal vacuoles as well as intracytoplasmic microvacuoles, most of which are dilated Golgi vesicles, and (2) light staining neurons of normal shape with clustering of organelles around the nucleus, i.e. "peripheral clearing" of the cytoplasm. The light type of change followed the same temporal sequence as in the cerebral cortex, i.e. it was seen during the hypoglycemia and it disappeared rapidly during restitution.

The dark type of nerve cell injury, however, followed a different temporal sequence: it did not appear in the caudoputamen until restitution was instituted, whereas in the cerebral cortex and hippocampus it was present already in brains fixed immediately after the hypoglycemic insult (3,8,9,18). The first dark neurons in the caudoputamen were detected after 5 min of recovery and their number increased for the next couple of hours. Recruitment of new, dark neurons seemed infrequent after 4-6 h. The transformation of dark neurons to acidophilic ones occurred, as in the cerebral cortex, during the first 2-8 h of recovery, i.e. in the period when EM results demonstrated alterations reflecting cell death. The results suggest that few, if any, dying or dead cells were recruited after that period, i.e. that the phenomenon of delayed neuronal death (15,21,22,27) did not occur. It should, however, be pointed out that in this series of experiments we focused our interest mainly on the qualitative aspects of the hypoglycemic cell

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injury. Thus, since statistically valid quantification of the damage at different time intervals was not attempted, our conclusions regarding the density of cell damage are provisional.

Fate of Irreversibly Injured Neurons and Tissue Reaction

Once they appeared, the course of the dark, condensed neurons in the caudoputamen was similar to that of the irreversibly damaged cortical and hippocampal pyramidal cells as described in the preceding articles (8,9). Thus, at the time when by LM transformation of dark neurons to acidophilic ones occurred EM showed that the nuclei of the severely affected ones became pyknotic with coarsely stippled chromatin, the plasma and nuclear membranes developed breaks, and flocculent densities appeared in mitochondria. Finally the neurons underwent karyorrhexis and cytolysis. The nerve cell destruction induced phagocytic reaction within one to two days, and after a few weeks macrophages were often the most numerous cells among the hypertrophic astrocytes and the few preserved neurons.

In the caudoputamen, like in the other areas of the brain, hypoglycemia caused selective neuronal necrosis, but frank infarction (i.e. destruction of all tissue components) did not occur. Nonetheless, vigorous astroglial reaction to the nerve cell destruction was seen. In the early period this was observed as perineuronal and perivascular glial swelling, later as hypertrophy of the astrocytes with abundant accumulation of cytoplasmic glial filaments. The dark change of the neurons did not seem to be directly related to development of edema in the surrounding astrocytes, since markedly condensed neurons were present already in minimally edematous areas (cf. Figs 2B and 8). However, as more

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cells became injured dense edema developed in close proximity to disintegrating neurons, suggesting that edema developed as a response to neuronal injury.

Pathogenesis of Dark Neurons

Having discussed data relevant to the questions posed in the introduction we deem it justified to consider the time course of the appearance of the dark neurons and conditions under which they occur. The pathogenetic mechanisms, which cause the condensation of injured neurons, are still enigmatic. Dark, condensed neurons have been described in several pathological conditions, ischemia and status epilepticus being two states closely related to hypoglycemia in the sense that they all are disorders of cerebral energy metabolism (e.g. 11,20,28). In complete cerebral ischemia, dark neurons are not observed until the blood flow is restored (5,16,17). This might indicate a connection between the dark neurons and restored energy production (cf. also appearance of dark neurons during status epilepticus, a condition which leaves cerebral energy state minimally perturbed). However, it does not explain the differences between the neocortex/hippocampus, and the caudoputamen during hypoglycemia, since no evidence is available that these structures differ in their metabolic and/or circulatory response to the hypoglycemic insult (1,2). Evidently, the mechanisms responsible for the dark neuron alteration remain enigmatic.

Comparison Between Hypoglycemia and Ischemia

As a final point we wish to compare cell injury in the caudoputamen during hypoglycemia and ischemia. We have already referred to the fact that the distribution of neuronal damage,

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although similar, is not identical in these two conditions (7). It remains to be discussed, whether the rate of cell death is similar. The LM data of Pulsinelli et al (26) indicate that the majority of cells destined to die do so within the first 3 h of recovery following transient four-vessel occlusion. Petito and Pulsinelli (25) reported that dark neurons with swollen mitochondria did not appear until after 15-30 min of reperfusion. The ischemic insult preferentially affected small and medium sized neurons, which at 3 h of reperfusion appeared as naked nuclei, whereas large neurons were preserved. Hence, the evolution of neuronal injury in the caudoputamen seems in many respects similar in hypoglycemia and ischemia.

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HYPOTENSION AS A COMPLICATION OF HYPOGLYCEMIA LEADS TO
ENHANCED ENERGY FAILURE BUT NO INCREASE IN NEURONAL NECROSIS

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ABSTRACT

The hypothesis that arterial hypotension aggravates hypoglycemic brain damage was tested. Thirty minutes of insulin-induced hypoglycemia with a flat EEG ("isoelectricity") was compared in seven series of rats. In three series of animals, the energy state of the cerebral cortex was determined at blood pressures of 140, 100 and 80 mm Hg respectively. Hypotension during hypoglycemia exacerbated cortical energy failure. In the fourth to sixth series, blood pressure was adjusted during isoelectricity to 160, 100 and 60 mm Hg, respectively. A seventh series had induced hypotension to 60 mm Hg only in the recovery period. Quantitation of neuronal death was performed in the fourth to seventh series of rats by direct visual counting of acidophilic neurons in sub-serially sectioned brains after one week survival. No augmentation of quantitated hypoglycemic neuronal necrosis was seen due to hypotension during or after hypoglycemic isoelectricity. The distinct distribution of hypoglycemic brain damage, suggesting a fluid-borne toxin, was present at normal and reduced blood pressures, with no tendency toward an ischemic pattern of pathology. In spite of previously demonstrated reductions of cerebral blood flow to ischemic levels in regions with pronounced loss of autoregulation, no regional exacerbation of neuronal necrosis was seen in these brain areas. It is concluded that hypoglycemic brain damage is distinct from ischemic brain damage, and that the two insults are not additive. Furthermore, moderate hypotension to 60 mm Hg does not aggravate the damage in spite of an enhanced energy failure.

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INTRODUCTION

Considerable information now exists on the neurochemical effects of severe insulin-induced hypoglycemia, as well as on the brain damage which may be incurred as a result of the hypoglycemia. Observations in man, and in animal experiments, demonstrate that stupor and coma, or their EEG equivalents, are accompanied by a reduced cerebral glucose utilization, at an unchanged or only slightly reduced oxygen consumption (1,2,3,4,5,6). Subsequent experimental studies gave detailed information on endogenous substrates utilized by the glucose-deprived brain (for literature, see Siesjö and Agardh (7)). These studies also showed that cessation of spontaneous EEG activity ("isoelectricity") occurs pari passu with deterioration of cerebral energy state, eg. with reduction in phosphocreatine (PCr) and ATP concentrations, and corresponding increases in ADP and AMP concentrations (8,9,10,11). The energy failure also leads to loss of ion homeostasis, since massive cellular efflux of K^+ (10) and uptake of Ca^{2+} (12,13) are observed.

Several observations suggest that hypoglycemia does not normally encroach upon cellular oxygen supply. Thus, studies designed to provide information on oxygen and glucose utilization (see above) showed that cerebral blood flow (CBF) is well maintained. Subsequent autoradiographic CBF measurements corroborated these observations (14,15). In fact, these studies showed that if blood pressure is maintained at values of 140-160 mm Hg, CBF is generally increased, in many structures to 200-300% of control (14,15). Furthermore, analysis of neurochemical variables suggest that cellular redox systems are oxidized during hypoglycemia (4,16).

All these studies allowed tight control of physiological variables such as blood pressure and arterial oxygenation. This has neither been the case in clinical material which has documented neuronal necrosis as a result of hypoglycemia (17,18,19,20,21), nor

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in earlier experimental studies confirming the clinical observations (22,23,24). Indeed, hypotension and/or hypoxia as contributing causes seemed likely. Thus, hypoglycemic and ischemic brain damage were considered identical (25,26,27,28,29), and hypotension was assumed to exacerbate hypoglycemic brain damage (28,29).

The advent of a physiologically controlled animal model which allows the delivery of purely hypoglycemic insults, followed by long term survival, has brought new information on the density and distribution of hypoglycemic neuronal necrosis (30,31). It has been shown that neuronal necrosis does not occur unless the EEG becomes isoelectric (30), suggesting that energy failure and loss of ion homeostasis are prerequisites for damage to be incurred. Furthermore, a comparison between hypoglycemic and ischemic insults in the same species demonstrates that the distribution of neuronal necrosis is not identical in the two conditions (31,32).

Although these results demonstrate that hypoglycemic brain damage can be incurred in the absence of cellular hypoxia, they do not provide an answer to the important question of whether arterial hypotension will aggravate hypoglycemic brain damage. Some recent results provide hints that this could be the case. Thus, experiments on cerebral acid-base regulation in severe hypocapnia (PaCO_2 15 mm Hg) showed that moderate hypotension further aggravated the deterioration of cerebral energy state, caused by hypoglycemia (33). Finally, it could be shown that vascular autoregulation was lost during hypoglycemia, and that even relatively moderate reductions in blood pressure could severely reduce local CBF (15).

With this background, we wished to test the hypothesis that hypoglycemic brain damage, defined as neuronal necrosis, is exacerbated by hypotension. Two series of animals were studied. In

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one, the influence of hypotension during hypoglycemia on the cortical energy state was investigated. To that end, blood pressure was held at 160, 100, and 80 mm Hg during a 30 min period of isoelectricity, and labile phosphates were analysed in neocortical tissue. In the second series, rats were subjected to a standard hypoglycemic insult at varying blood pressures, and were then allowed to recover one week before perfusion fixation, followed by quantitative histopathological examination.

MATERIALS AND METHODS

Seventy six male Wistar rats weighing 290-345 g were used (Møllegaard Breeding Center, Copenhagen). The animals for the metabolite series were divided into groups with high (group A, about 140 mm Hg), medium (group B, 100 mm Hg), and low (group C, 80 mm Hg) blood pressures. At each pressure, brains were frozen in situ for metabolite measurements after 5 and after 30 min isoelectricity. Normoglycemic control brains were similarly analysed at 140-160 mm Hg and 100 mm Hg.

The animals for the histopathology series were also divided into high (D), medium (E), and low pressure (F) groups. However, to ensure that the high pressure group had high cerebral perfusion rates, the animals were regulated to a pressure of at least 140 mm Hg. Furthermore, to ensure that the low pressure animals had enhanced deterioration of cerebral energy state, they were kept at a blood pressure of 60 mm Hg during the period of isoelectricity.

A seventh series (G) was also examined by quantitative histopathology. Here the blood pressure was held at 140 to 160 mm Hg during the isoelectric period, but was lowered to 60 mm Hg in the recovery period.

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A previous publication describes more completely the model used (30). Briefly, the rats were fasted overnight with free access to tap water. The next day, they were given 9-21 I.U./kg of regular insulin (Actrapid, Novo Industri A/S, Copenhagen), immersed in 3% halothane, intubated, and mechanically ventilated with 0.8% halothane in a 2:1 N₂O:O₂ mixture. Two tail veins, and the tail artery were then cannulated, and continuous paralysis maintained via an i.v. infusion of suxamethonium. A bipolar interhemispheric electroencephalogram (EEG) was continuously monitored. The halothane was discontinued, the animals were ventilated on a 2:1 N₂O:O₂ mixture, and a flat EEG ("isoelectricity") was awaited.

During isoelectricity, blood pressure was held at the desired level through controlled exsanguination and re-infusion of blood. The actual blood pressures throughout the period of isoelectricity are given in Fig. 1 for the histopathology series D to F. The temperature, arterial PO₂, PCO₂, and pH were maintained within the physiological range.

The animals in which the energy metabolites were studied had a funnel fitted onto the skull. The brain was then frozen in situ according to Pontén et al (34). After removal of the brain from the skull it was kept at -80°C until analyzed. Fragments weighing approximately 25 mg were dissected from the parietal cortex at -20°C, and were extracted in HCl-methanol and subsequently in perchloric acid at +0°C. After neutralization, the metabolites were determined fluorometrically (35). The conditions for the analyses have been previously described (36,37).

After thirty minutes of EEG isoelectricity the animals in the histopathology series were recovered with an infusion of 0.2 ml of 50% glucose, given by hand over one minute. A 50% glucose infusion was then tapered over the ensuing hours, and substituted with 25% glucose in Krebs-Henseleit solution overnight, in order to maintain

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a plasma glucose of between 5 and 10 mM/l. Following extubation, usually one to three hours after glucose induced recovery, blood glucose, blood gases and pH were checked finally in the awake, conscious animal before removal of the tail catheters. The animals were weighed and examined daily, and were allowed to survive one week. They were sacrificed by perfusion fixation with 4% phosphate buffered formaldehyde under 1% halothane anesthesia. The brains were left in situ at 4°C and were removed the following day. After processing in graded ethanols and xylol, they were embedded in paraffin and sub-serially sectioned at 8 μ m.

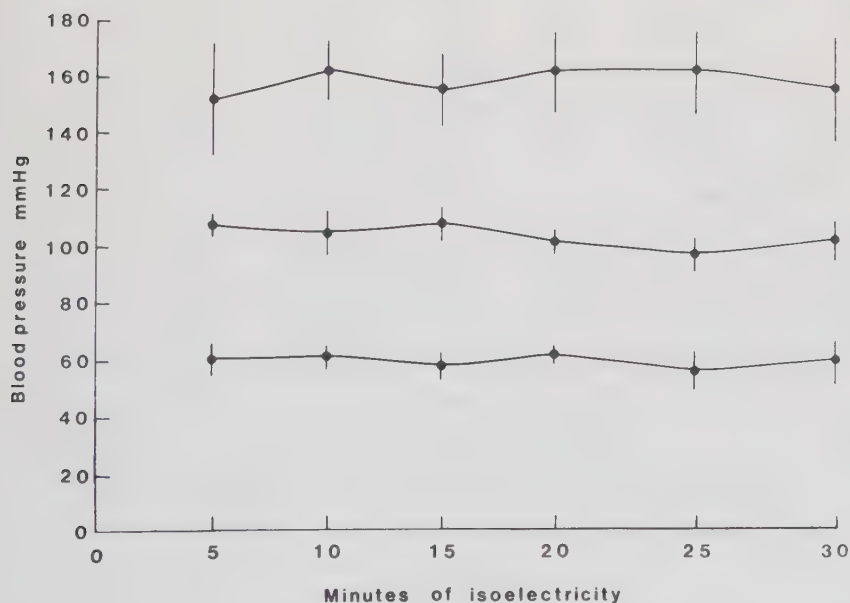


Figure 1. Blood pressure as a function of duration of isoelectricity for each group. All values are mean $\pm$ standard deviation.

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Sections were double stained with acid fuchsin and cresyl violet, and the number of acidophilic neurons was assessed by direct visual counting of sections at standardized coronal levels of the brain (30).

The crude damage index (CDI) was generated as follows: For the caudate nucleus, subiculum, CA1 pyramidal neurons, and dentate granule neurons, the following numbers were assigned for the per cent neuronal necrosis: <10% = 1, 10-50% = 2, and 50-100% = 3. For the cerebral cortex, 10 to 100 necrotic neurons per section was assigned the number 1, 100 to 1000 the number 2, and >1000 the number 3. These integers were added to produce the CDI for each brain.

RESULTS

Physiologic parameters during EEG isoelectricity are shown in Table 1. All animals were normothermic and normoxic, and their PCO₂ and pH values showed little deviation from control. Results from the metabolite series demonstrate that mean arterial blood pressure was kept constant between the 5th and the 30th minute. Corresponding blood pressure data from the histology series are shown in Fig. 1. The blood pressure in the recovery period in the post-insult hypotension group was comparable to that shown during isoelectricity in the low blood pressure group.

1. Cerebral Metabolites

Cerebral metabolic changes were similar after 5 and 30 min of isoelectricity, demonstrating that a new steady state is reached soon after cessation of EEG activity (11,38). Due to this constancy, we will discuss the 30 min data only. Furthermore, since

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Table 1

Physiologic parameters during hypoglycemia. The values are taken at the end of the given EEG isoelectric period.

Group	No of animals	MABP (mm Hg)	Temp (°C)	PO ₂ (mm Hg)	PCO ₂ (mm Hg)	pH	Blood glucose (mM)
Metabolites							
*Control 1	8	136 _{±9}	37.0 _{±0.6}	103 _{±11}	35.4 _{±1.2}	7.35 _{±0.05}	
*Control 2	6	96 _{±8}	37.2 _{±0.5}	97 _{±8}	32.9 _{±1.5}	7.39 _{±0.04}	
A 5'	6	143 _{±6}	37.0 _{±0.2}	100 _{±9}	32.0 _{±1.6}	7.34 _{±0.04}	
A 30'	6	142 _{±4}	37.0 _{±0.3}	101 _{±7}	34.5 _{±3.0}	7.34 _{±0.04}	
B 5'	6	101 _{±2}	36.8 _{±0.4}	108 _{±10}	30.5 _{±10.4}	7.36 _{±0.17}	
B 30	4	101 _{±6}	37.0 _{±0.3}	110 _{±20}	32.9 _{±2.1}	7.31 _{±0.07}	
C 5'	6	80 _{±0}	36.9 _{±0.3}	106 _{±9}	37.1 _{±4.4}	7.29 _{±0.09}	
C 30'	8	79 _{±2}	37.0 _{±0.3}	105 _{±5}	32.0 _{±2.7}	7.29 _{±0.03}	
Histology							
D	6	159 _{±12}	37.3 _{±0.3}	93 _{±24}	37.2 _{±4}	7.38 _{±0.05}	0.53 _{±0.17}
E	8	104 _{±2}	37.3 _{±0.2}	105 _{±10}	35.7 _{±2}	7.37 _{±0.05}	0.41 _{±0.15}
F	6	60 _{±2}	37.3 _{±0.2}	94 _{±6}	33.8 _{±3}	7.41 _{±0.06}	0.35 _{±0.09}
G	6	63 _{±6} **	37.5 _{±0.4}	109 _{±9}	37.4 _{±4}	7.38 _{±0.06}	0.31 _{±0.12}

All values are given as mean $\pm$ standard deviation.

*In the metabolite group, control 1 denotes a normoglycemic group with spontaneous blood pressure (140 to 160 mm Hg), and control 2 denotes a normoglycemic group exsanguinated to a blood pressure of 100 mm Hg

**Mean arterial blood pressure in the first 30 min of recovery

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Table 2

Cerebral cortical concentrations of labile energy metabolites during insulin induced hypoglycemia in animals subjected to different blood pressures during the hypoglycemic period.

Group	Glucose	PCr	ATP	ADP	AMP	Energy Charge	Lactate	Pyruvate
Control	2.25 $\pm$.39	4.46 $\pm$.18	2.88 $\pm$.04	.275 $\pm$.008	.066 $\pm$.012	.937 $\pm$.003	1.51 $\pm$.11	.108 $\pm$.007
A (High BP 160)	.07 $\pm$.03	.57 $\pm$.11	.81 $\pm$.11	.915 $\pm$.091	.447 $\pm$.025	.580 $\pm$.026	.41 $\pm$.09	.036 $\pm$.002
B (Med BP 100)	.14 $\pm$.11	.50 $\pm$.08	.72 $\pm$.14	.821 $\pm$.075	.575 $\pm$.056	.532 $\pm$.027	.38 $\pm$.08	.019 $\pm$.005
C (Low BP 80)	.03 $\pm$.01	.22 $\pm$.05	.36 $\pm$.05	.724 $\pm$.028	.768 $\pm$.027	.307 $\pm$.021	.33 $\pm$.04	.022 $\pm$.002

The values are given in $\mu\text{mol g}^{-1}$ wet weight and represent means $\pm$ standard error of the mean.

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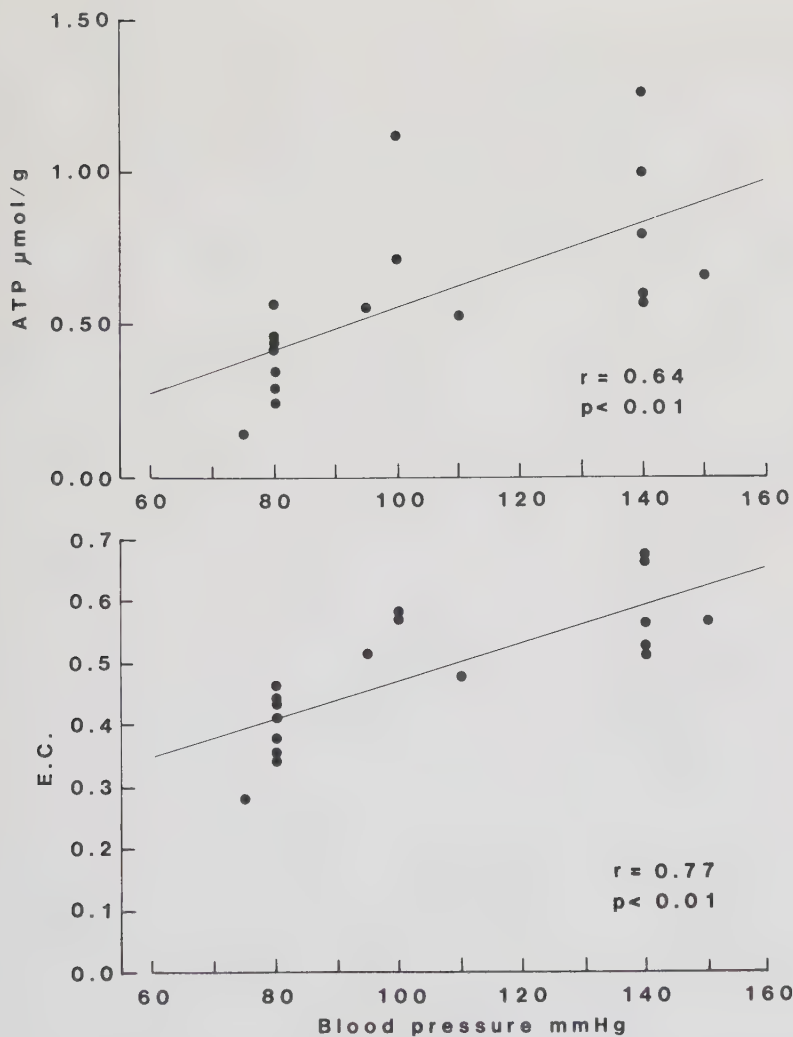


Figure 2. Tissue level of ATP and energy charge as a function of blood pressure in animals subjected to 30 min of hypoglycemia with an isoelectric EEG.

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the hypotensive control animals had values identical to normotensive ones, only the latter are shown in Table 2, which gives tissue metabolites. In all isoelectric animals, tissue glucose concentrations were very low. In the normotensive group, PCr and ATP concentrations decreased to about 10% and about 25% of control, respectively, with corresponding increases in ADP and AMP concentrations. As expected, lactate and pyruvate concentrations were reduced.

The results of Table 2 demonstrate that hypotension further reduced PCr and ATP concentrations, as well as the calculated adenylate energy charge, as defined by Atkinson (39). The cortical energy charge during hypoglycemia was 0.58 at a blood pressure of 140 mm Hg, 0.53 at 100 mm Hg, and sank to 0.31 at 80 mm Hg. In order to illustrate the pressure-dependent deterioration of cerebral energy state, individual values for ATP and energy charge were plotted against blood pressure (Fig. 2). The results demonstrate a significant correlation ($p < 0.01$). At a blood pressure of 80 mm Hg, PCr and ATP concentrations were reduced to 5 and 10% of control respectively.

2. Histopathology

The density of neuronal necrosis was determined by direct visual cell counts in each of the major brain regions. The data are summarized¹ in Table 3. As the results demonstrate, no effect of hypotension in enhancing neuronal necrosis was seen. The results obtained in the group maintained hypotensive for 30 min in the immediate recovery period were likewise negative.

Figure 3 plots the crude damage index, derived from the number of necrotic neurons in each brain region (see Material and Methods)

¹Tabulated data from each animal may be obtained if requested by letter to Dr. Auer.

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against the blood pressure. It is apparent that there was considerable variation in brain damage within each group, but the degree of damage was unchanged by lowering the blood pressure to either 100 mm Hg or 60 mm Hg.

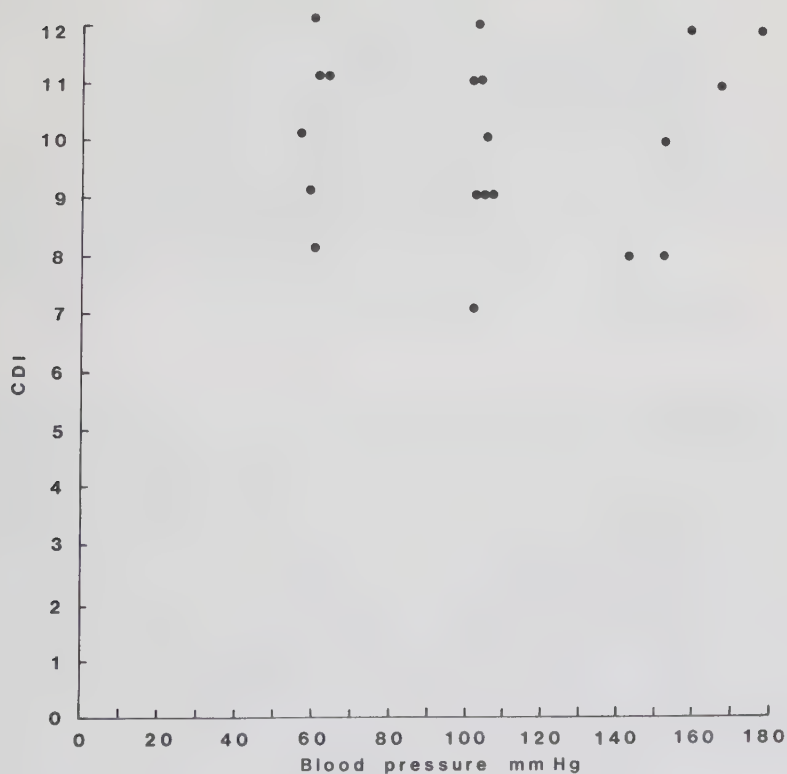


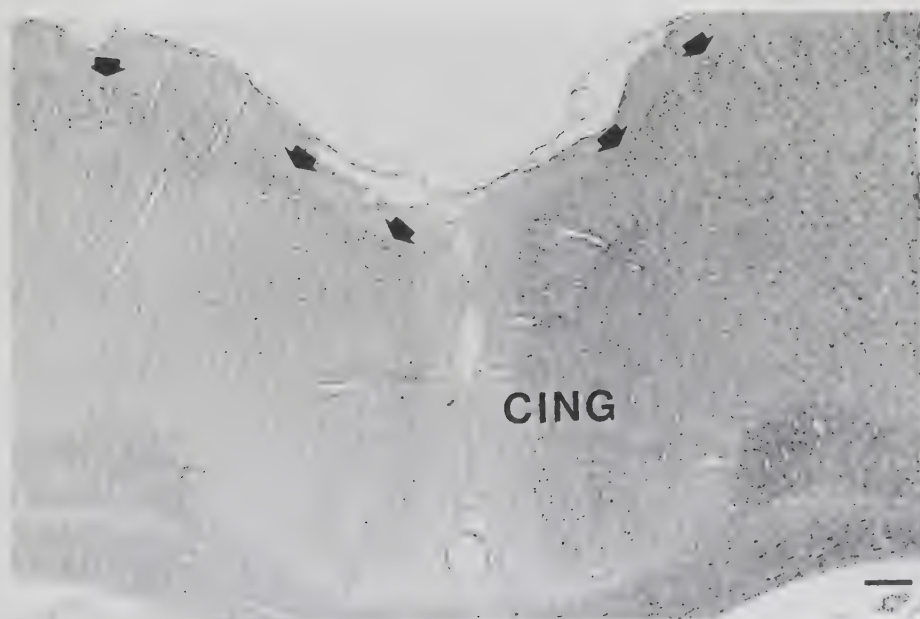
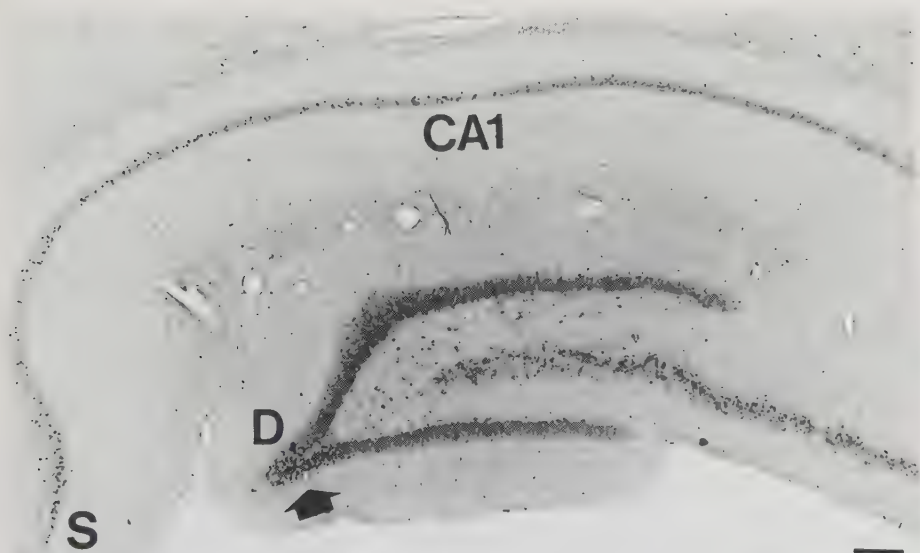
Figure 3. Crude damage index (see p 176) as a function of mean BP during 30 min isoelectricity. Neither lowering the BP to 100, nor to 60 had an effect on the quantitated brain damage.

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Figure 4. Histologic appearance of hippocampal damage, one week after 30 min of hypoglycemic isoelectricity complicated by hypotension to 60 mm Hg. The distribution is identical to that seen after hypoglycemic isoelectricity accompanied by a BP of 100 or 160. There is neuronal necrosis at the crest (arrow) and external blade of the dentate gyrus (D), and in the CA1 pyramidal cells (CA1), especially medially near the subiculum (S).
Acid fuchsin/Cresyl violet. Bar = 200 μ m.

Figure 5. The cingulate gyri (CING), structures showing marked loss of autoregulation, and a consequent drop in blood flow to ischemic levels during hypoglycemia accompanied by hypotension to 60 mm Hg, nevertheless show relative sparing compared to the neuronal necrosis seen in the surrounding superficial cortex (arrows).
Acid fuchsin/Cresyl violet. Bar = 250 μ m.

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Table 3

Summary of Histologic Data

Group	Caudate %	Dentate %	CA1 %	Subiculum %	Cortex, cells
D	79 $\pm$ 6	14 $\pm$ 3	17 $\pm$ 8	77 $\pm$ 7	145 $\pm$ 78
E	83 $\pm$ 7	19 $\pm$ 5	11 $\pm$ 7	88 $\pm$ 5	115 $\pm$ 37
F	80 $\pm$ 6	15 $\pm$ 4	26 $\pm$ 6	93 $\pm$ 3	201 $\pm$ 85
G	78 $\pm$ 7	12 $\pm$ 6	21 $\pm$ 13	82 $\pm$ 9	109 $\pm$ 71

All values mean $\pm$ standard error of the mean.

No statistically significant differences were noted.

Not only the density of brain damage, but also the distribution of hypoglycemic brain damage was unaltered by lowering the blood pressure to 100 or to 60 mm Hg. Affected areas included the dentate gyrus (Fig. 4), the cerebral cortex (Fig. 5) and septal nuclei (Fig. 6) in a pattern identical to that seen after hypoglycemia at a blood pressure of 160 mm Hg. Thus, the crest and the external blade of the dentate gyrus were affected first. The superficial cerebral cortical layers showed the most damage, with a superficial to deep gradient seen. The cerebellum was affected near the foramina of Luschka, with numerous necrotic Purkinje cells seen over the hemispheres in some animals (not shown). Infarction was never seen.

No features regarding the distribution of neuronal necrosis serve to distinguish the brain damage observed in the present study from that previously reported (31). However, due to the marked differences in the degree of remaining autoregulation, special attention was focussed on certain brain regions histologically. Notably, the cingulate gyrus, known to have an ischemic flow rate during hypotensive hypoglycemia as studied here (15), showed

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sparing, rather than enhancement of neuronal necrosis (Fig. 5).

Conversely, the septal nuclei show relative preservation of autoregulation (15), but nevertheless demonstrated selective neuronal necrosis histologically (Fig. 6).

SEPTAL NUCLEI

A black and white micrograph of a brain section, specifically the septal nuclei. The image shows a dense population of small, dark-stained cells. Several arrows point to specific areas of neuronal necrosis. A horizontal scale bar is located in the lower right corner of the image.

Figure 6. The septal nuclei, a brain region showing relative preservation of autoregulation during hypoglycemia, consequently undergoing only minor decreases in blood flow secondary to hypotension, nevertheless show neuronal necrosis (arrows).

Acid fuchsin/Cresyl violet. Bar = 500 μ m.

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DISCUSSION

Hypotension has long been assumed to exacerbate hypoglycemic brain damage (28,29). The present experiments were designed to test this hypothesis, and are based on an animal model which allows tight control of physiologic parameters, as well as long term survival. The former was considered necessary to unequivocally dissect out cause and effect relationships between the physiologic and metabolic data during and after the insult, and the consequent neuronal damage. The latter was deemed necessary to allow for the unequivocal signs of neuronal necrosis to develop.

The results demonstrate a very marked dichotomy. Thus, in view of the results of the metabolic part of this study, the finding of no effect of hypotension to 60 mm Hg either during or after the period of isoelectric EEG on either the density or the distribution of hypoglycemic neuronal necrosis is somewhat surprising.

As stated in the introduction, results obtained in severely hypocapnic animals demonstrated a relationship between blood pressure and the adenylate energy charge of neocortical tissue, suggesting that even moderate hypotension will aggravate the deterioration of cellular energy metabolism during hypoglycemia (40). Results obtained with measurements of extracellular K^+ activity were in line with this conclusion (33). These results, and those showing loss of vascular autoregulation in many brain structures during hypoglycemia (15), suggest that hypotension leads to a reduction in flow rate of sufficient magnitude to further reduce glucose delivery to the substrate-deprived cells, or to curtail oxygen supply.

It is of considerable interest that a reduction of ATP concentration to $0.36 \pm 0.05 \text{ mol} \cdot \text{g}^{-1}$ of tissue, as occurred in the group C, did not aggravate hypoglycemic neuronal necrosis. In fact, since the histopathology group F had an even lower blood pressure

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(60 mm Hg) than metabolite group C (80 mm Hg), it would seem that reduction of ATP concentration toward zero causes no aggravation of the neuronal damage. The results suggest that the events which lead to hypoglycemic neuronal necrosis occur already at a normal or reduced blood pressure. Such events include a reduction of ATP concentration to about 25% of control, marked increases in ammonia concentrations (1), aspartate (41) and in free fatty acid concentrations (42), massive depolarization of cells with release of K^+ (10,33), and reductions of extracellular Ca^{2+} concentration to very low levels, probably because of influx into cells (12,13).

It now seems established that hypoglycemia leads to neuronal necrosis with a different distribution than that observed in ischemia (30,31,32). The ischemic pattern of distribution shows involvement of the middle cortical layers, uniform involvement of the CA1 pyramidal cell band of the hippocampus, but conspicuous sparing of the dentate gyrus until damage is very severe (32).

The hypoglycemic pattern of distribution involves an affection of the superficial layers (2-3) of neocortical cells, medial CA1 pyramidal cells of the hippocampus, and heavy affection of cells at the crest of the dentate gyrus, the conspicuous relationship of neuronal necrosis to tissue fluid and CSF pathways suggesting the participation of a fluid-borne toxin (31). Dark neurons are seen acutely, and acidophilic neurons subsequently, in all these brain regions (43,44,45). Although the dark neurons may potentially recover (43), the acidophilic neurons, as seen in the present study, are established as necrotic by their mitochondrial flocculent densities and absent cell membranes, and by their subsequent removal from the tissue (43,44,45).

Since the distribution of neuronal necrosis is not the same in ischemia and hypoglycemia, it is possible to distinguish hypoglycemic and ischemic patterns of brain damage. Lowering the

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blood pressure during, and after the period of hypoglycemic isoelectricity might theoretically have given rise to an ischemic pattern of brain damage, a hypoglycemic pattern of brain damage, or even a combination of the two. It is of interest that hypotension did not alter the pattern of distribution of neuronal necrosis from one typical of hypoglycemia to one more characteristic of ischemia. Since the density of hypoglycemic brain damage was also unaffected by hypotension, one may conclude that formation of the hypothetical toxin is not enhanced when ATP concentrations are further reduced.

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THE DENTATE GYRUS IN HYPOGLYCEMIA:
PATHOLOGY IMPLICATING EXCITOTOXIN MEDIATED NEURONAL NECROSIS

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ABSTRACT

A detailed light and electron microscopic study of the damage to the rat dentate gyrus in hypoglycemia was undertaken, in view of the previously advanced hypothesis that hypoglycemic nerve cell injury is mediated by a released neurotoxin. The distribution of neuronal necrosis showed a relationship to the subarachnoid cisterns.

Electron microscopy of the dentate granule cells and their apical dendrites revealed dendro-somal, axon-sparing neuronal pathology. Dentate granule cells were affected first in the dendrites in the outer layer of the stratum moleculare, sparing axons of passage and terminal boutons. Subsequently, the neuronal perikarya were affected, and Wallerian degeneration of axons followed. Cell membrane abnormalities preceded the appearance of mitochondrial flocculent densities and degradation of the cytoskeleton, and are suggested to be early lethal changes.

The observed early dendrotoxic changes, and the dendro-somal, axon-sparing nature of the lesion implicate an excitotoxin mediating neuronal necrosis in hypoglycemia.

INTRODUCTION

The dentate gyrus is a portion of the hippocampus prone to undergo selective neuronal necrosis in hypoglycemia (4,5,22,25,47,51), this being one of the features distinguishing the neuropathology of hypoglycemic brain damage from ischemic brain damage. The distribution of neuronal necrosis in hypoglycemic brain damage has been shown to have a relationship to the white matter and cerebrospinal fluid pathways of the brain (5). The pattern of

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labelling of dentate granule neurons by subarachnoid horseradish peroxidase (11) is similar to the pattern of final neuronal necrosis seen in hypoglycemia (5).

A hypothesis has therefore been proposed that the nerve cell injury seen in hypoglycemia is in some way mediated by a factor in the extracellular fluid in and around the brain, influencing the cell membrane or the cytoplasmic organelles (5,47). The present work was undertaken in view of this hypothesis, and the distribution of neuronal necrosis within the dentate gyrus was thus studied in detail. In addition, to define the temporal sequence of cytopathologic changes in neuronal death in the at the dentate crest, a location which regularly undergoes neuronal necrosis, the dendritic fields, cell bodies, and axons of the dentate cells were studied through closely spaced time intervals by light and electron microscopy.

The results support the hypothesis that a neurotoxin is present in the extracellular space in and around the brain, and the dendro-somal, axon-sparing nature of the lesion suggests the compound is an excitotoxin.

MATERIAL AND METHODS

Animal Model

A previous publication describes more completely the model used (4). Briefly, 46 male Wistar rats were fasted overnight with free access to tap water. The next day, they were given 9-22 I.U./kg of regular insulin (Actrapid, Novo Industri A/S, Copenhagen), anaesthetised with 3% halothane, intubated, and mechanically ventilated with 0.8% halothane in a 2:1 N₂O:O₂ mixture. Two tail veins, and the tail artery were then cannulated, and continuous paralysis maintained via an i.v. infusion of suxamethonium. Blood

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pressure was held at 150 ± 10 mm Hg by controlled exsanguination and re-infusion of blood. The temperature, arterial PO_2 , PCO_2 , and pH were maintained within the physiological range. A bipolar interhemispheric electroencephalogram (EEG) was continuously monitored. The animals were ventilated on a 2:1 $N_2O:O_2$ mixture and cerebral isoelectricity was awaited.

After 10 to 60 min of EEG isoelectricity, recovery was induced with an infusion of 0.2 ml of 50% glucose, given by hand over one min. A 50% glucose infusion was then tapered over the ensuing hours, and substituted with 25% glucose in Krebs-Henseleit solution overnight, in order to maintain a plasma glucose of between 5 and 10 mM. Following extubation, usually one to three hrs after glucose induced recovery, blood glucose, blood gases and pH were checked finally in the awake, conscious animal before removal of the tail catheters. The animals were weighed and examined daily, and were perfused after hypoglycemia with delta wave EEG slowing only (control), after 10, 20, 30, and 60 min of isoelectricity without recovery, and after 5, 10, 30, 60 min, 2, 3, 4, 6, 8, 12, 18, 24 hr, 2, 3, 5, and 7 days in rats after 30 and 60 min isoelectricity, and after 2, 3, and 6 weeks, 3, 6, and 12 months of recovery after 30 min of cerebral isoelectricity. One or two animals were studied at each of these time intervals. The material derives from the experiments performed to study the cerebral cortex (6) and hippocampus pyramidal cell band (7).

Light and Electron Microscopic Procedure

The rats were sacrificed by the intra-aortic perfusion of 4% formaldehyde for light microscopy, or 3% glutaraldehyde for electron microscopy and high resolution light microscopy, in 0.1 mol/l phosphate buffer (pH 7.4), warmed to 37°C. This was performed

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via thoracotomy on the tracheostomized or re-intubated rat while on a respirator and under 0.8% halothane. After administration of 90 I.U. intracardiac heparin, the descending aorta was clamped, the cerebral vasculature was washed out with isotonic saline, and 400 ml of fixative was allowed to perfuse through the cerebral circulation from a height of 180 cm, taking approximately 20 min. The brain was allowed to fix in situ, and was removed the following day.

For the light microscopic study of the distribution of neuronal necrosis, performed after one week survival, the brains were sectioned coronally into 2.8 mm. thick slices, dehydrated in graded ethanols, embedded in paraffin, sectioned at 8 μ m, and stained with 1% acid fuchsin and 5% celestine blue.

For the study of the temporal sequence of cytopathologic changes after the time intervals listed above, tissue was embedded in a soft methacrylate plastic polymer (EFL-67, Serva Feinbiochemica, Heidelberg, F.R.G.), sectioned at 1.5 μ m and stained with 1% acid fuchsin and 0.05% toluidine blue.

For electron microscopy, portions of the dentate gyrus were dissected out, post-fixed in 1% OsO₄, dehydrated, and embedded in Epon 812. Thin sections were selected from the stratum moleculare superjacent to the crest of the gyrus (Fig. 1), from the granule cell layer, and from the dentate hilus (Fig. 2), after the survival intervals noted above. They were examined in a JEOL JEM100C electron microscope.

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RESULTS

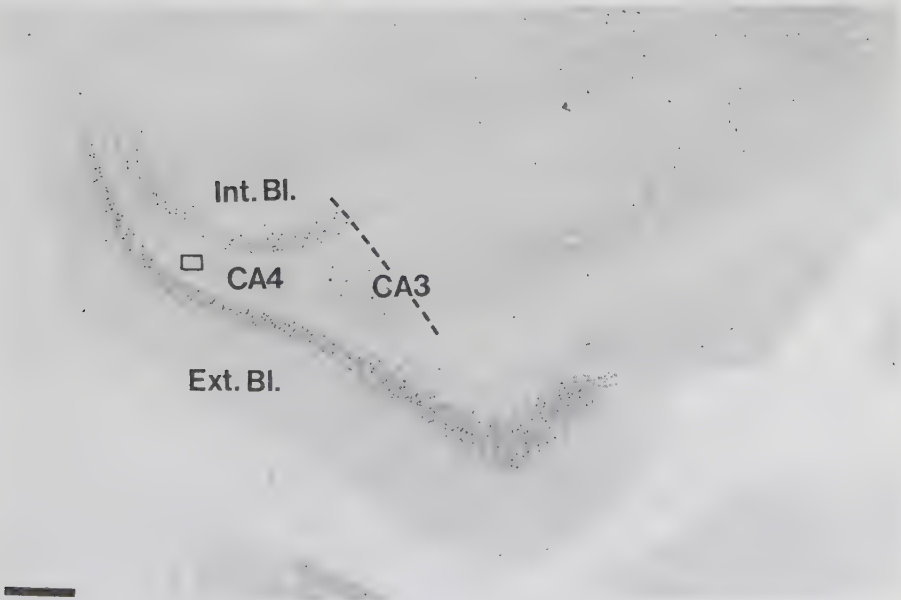
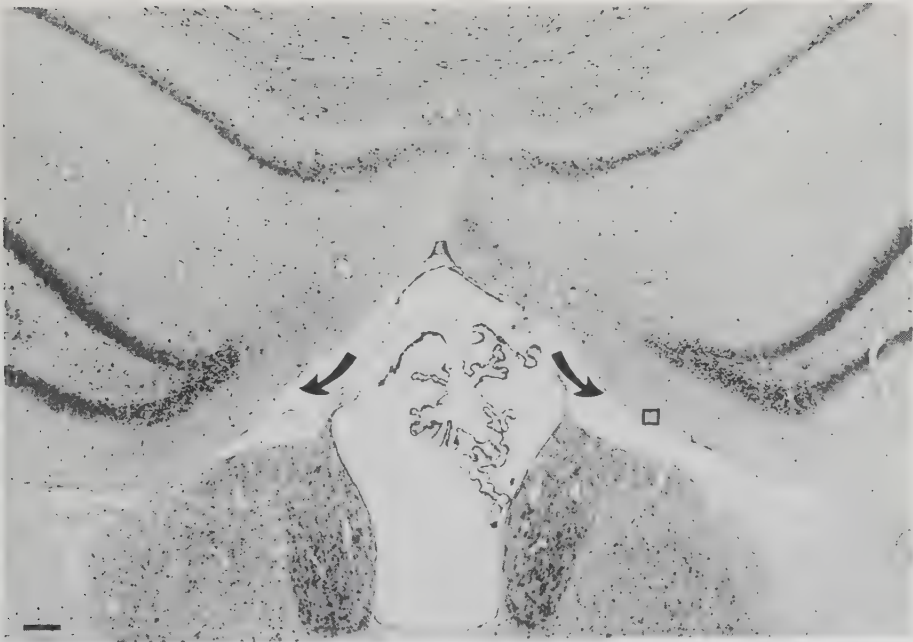
Light Microscopy

After one week survival, the damage at mid septo-temporal levels of the dentate gyrus was localised to the neurons beneath the subarachnoid cistern above the third ventricle (Fig. 1). With more severe damage (Fig. 2), necrosis spread to involve the entire

Figure 1. Dentate gyri at mid septo-temporal level. Only neurons with dendritic trees in the stratum moleculare exposed to the open cistern are affected. The portions of the gyrus apposed to the diencephalon are spared. Normal pathway of CSF flow from corpus callosum is indicated by arrows. Location of E.M. samples taken for figure 4 indicated in rectangle. 30 min isoelectricity, 1 week survival. Acid fuchsin/Cresyl violet. Bar = 200 μ m.

Figure 2. With severe damage, the entire external blade (Ext.Bl.) shows neuronal necrosis, and damage has spread along the internal blade (Int.Bl.) adjacent to the hippocampal fissure. CA4, and a portion of CA3 are affected, forming a front of damaged neurons to the left of the dashed line. Area sampled in figure 8 indicated in rectangle. 60 min isoelectricity, 1 week survival. Acid fuchsin/Cresyl violet. Bar = 200 μ m.

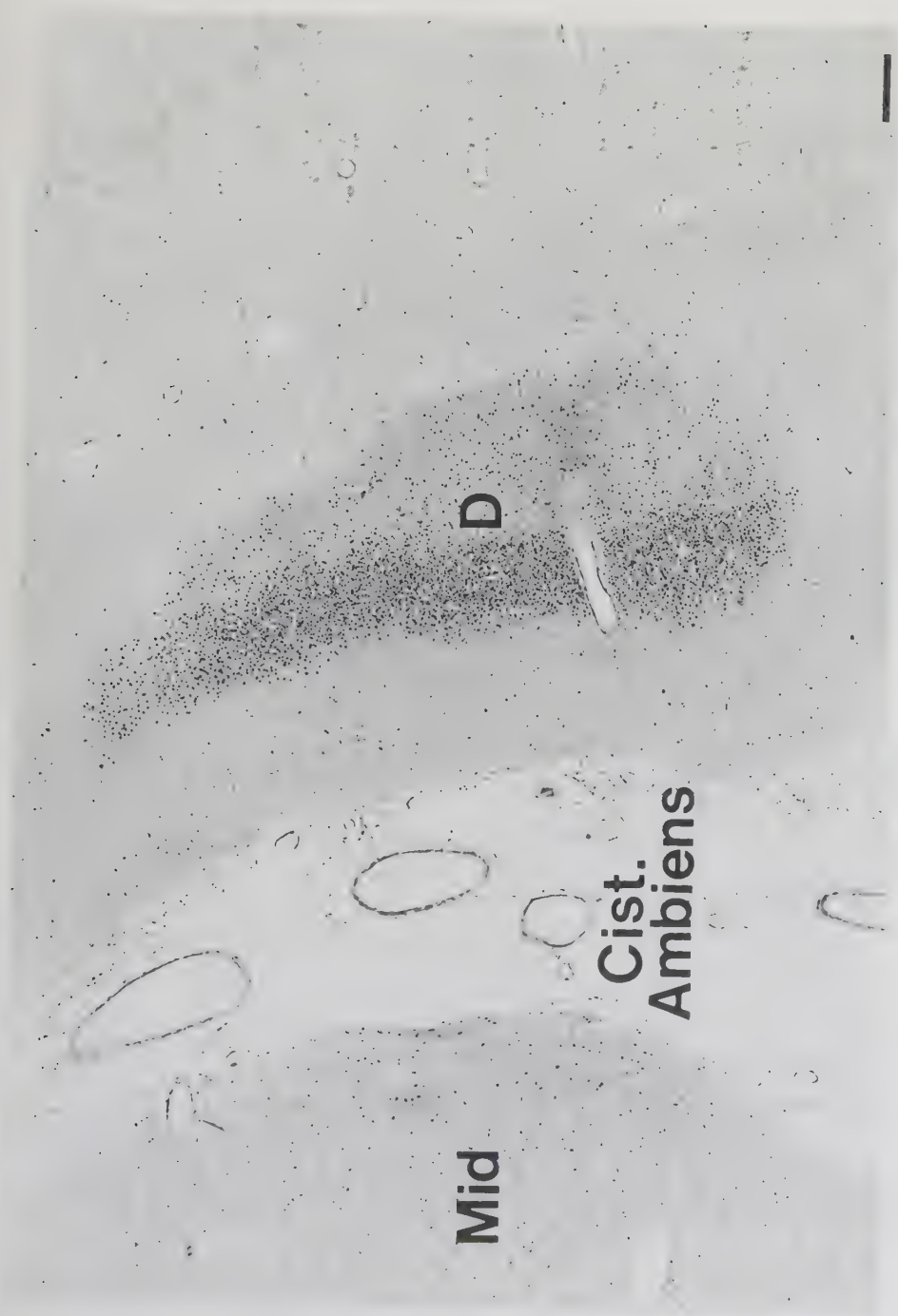
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Figure 3. Dentate (D), in temporal portion of hippocampus, at the level of the midbrain (Mid), where the gyrus appears as a distorted oval in coronal sections. There is dense neuronal necrosis of cells with dendrites facing the cisterna ambiens. 60 min isoelectricity, 1 week survival. Acid fuchsin/Cresyl violet. Bar = 200 μ m.

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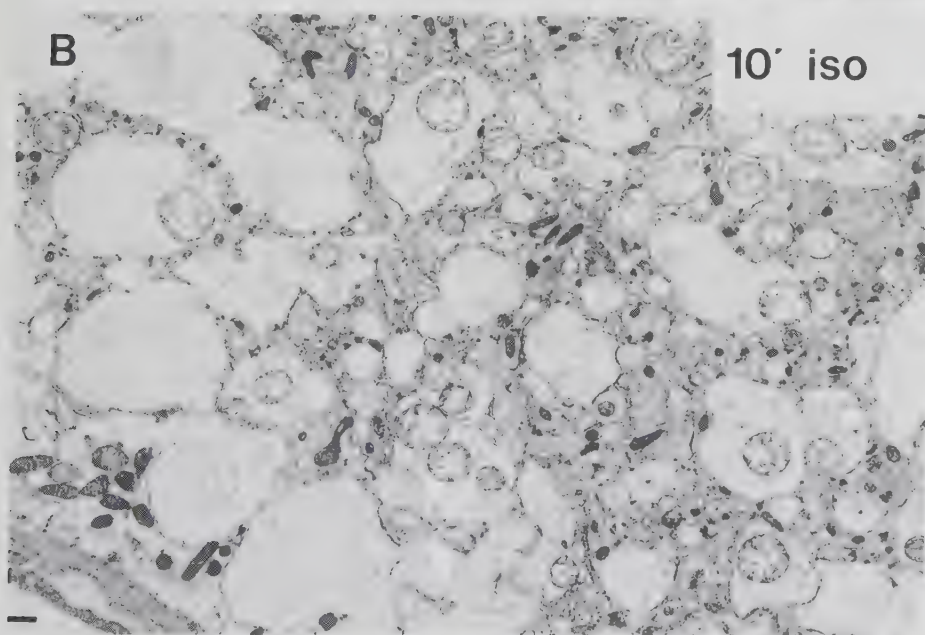
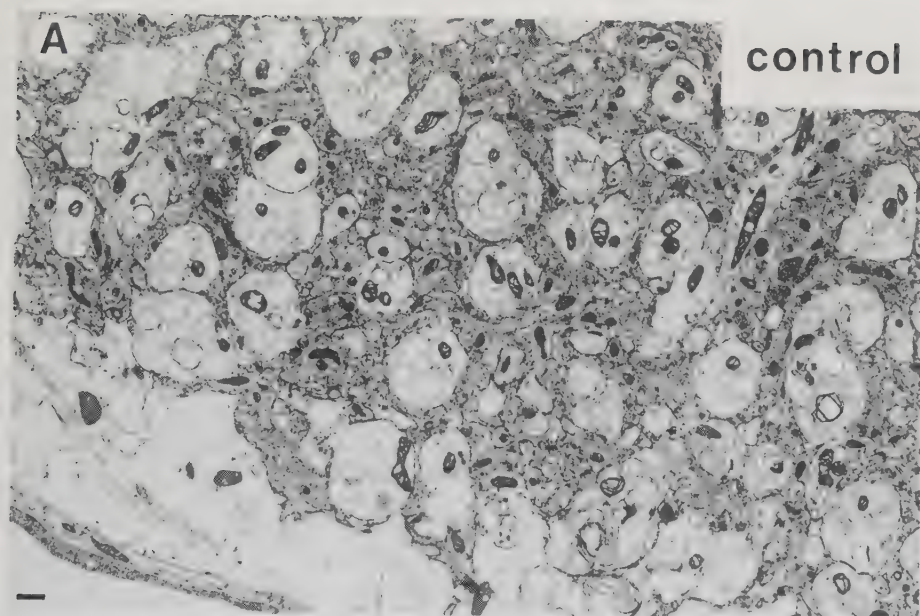


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Figure 4. Superficial stratum moleculare of the dentate from the location indicated in figure 1. The subarachnoid space is at the lower left of each picture.

- A. Control animal, fixed during EEG slowing, already shows swelling of dendrites, but dark, compact mitochondria in the entire neuropil.
 - B. After 10 minutes of cerebral isoelectricity, the dendrites are extremely swollen and appear ruptured. Numerous ballooned mitochondria with fractured cristae are seen in swollen neuronal process, but intervening processes contain normal compact and dark mitochondria.
- Bar = 1 μ m.

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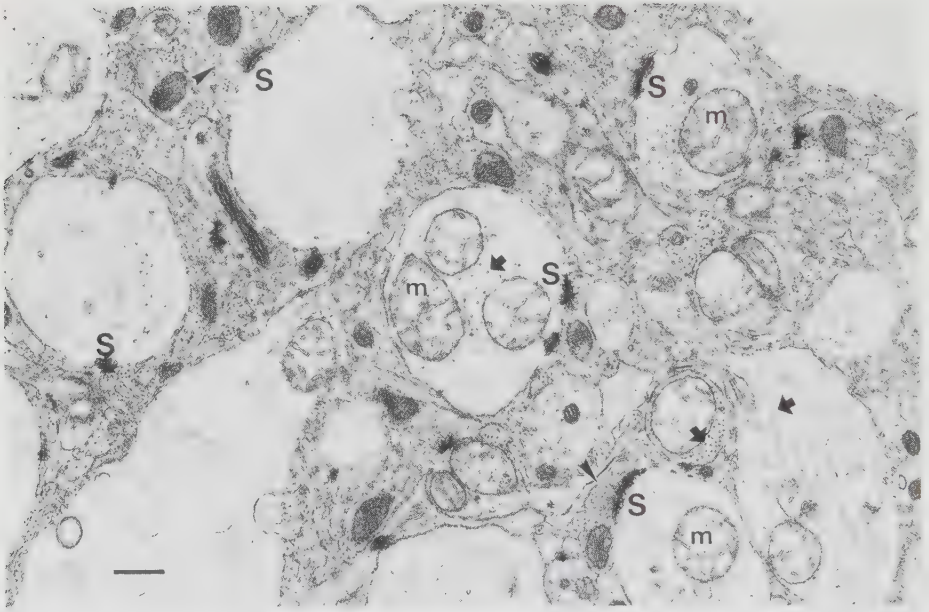


Figure 5. Swollen processes are revealed to consist solely of dendrites, as indicated by synapses (S), as well as predominance of neurotubules (arrows) over neurofilaments. Synaptic vesicles (arrowheads) are present on the opposite side of the synapse from the dendrites, in the axonal boutons terminaux. Axons and their contained mitochondria are spared, but dendritic mitochondria (m) are swollen. Stratum moleculare, 10 minutes after onset of isoelectricity. Bar = 1 μ m.

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external blade and most of the internal blade. The most severely affected animals, one week after 60 min isoelectricity, showed necrosis of the subjacent CA4 neurons in the dentate hilus, and finally CA3 pyramidal cells, forming a front.

The septal end of gyrus, open to the subarachnoid space, showed damage along the entire granule cell band. At temporal levels, sharply demarcated neuronal necrosis was seen in one half of the gyrus: Neurons with dendrites in the stratum moleculare facing the cisterna ambiens were necrotic. Those with dendrites in the stratum moleculare under the fused hippocampal fissure were spared (Fig. 3).

The temporal evolution of light microscopic changes was identical to that previously described in the cerebral cortex (6) and CA1 pyramidal cells of the hippocampus (7). Briefly, dark neurons appeared at the dentate crest after 30 min cerebral isoelectricity, and underwent transformation into acidophilic neurons after one to two hrs, coinciding with appearance of mitochondrial flocculent densities and the other ultrastructural features of cell death under the electron microscope (see below). Subsequent tissue changes involved removal of cytorrhectic neurons after several weeks. Infarction was never seen.

Electron Microscopy

Control material fixed during delta wave slowing of the EEG already showed dendritic swelling (Fig. 4A). This was not seen in normal animals.

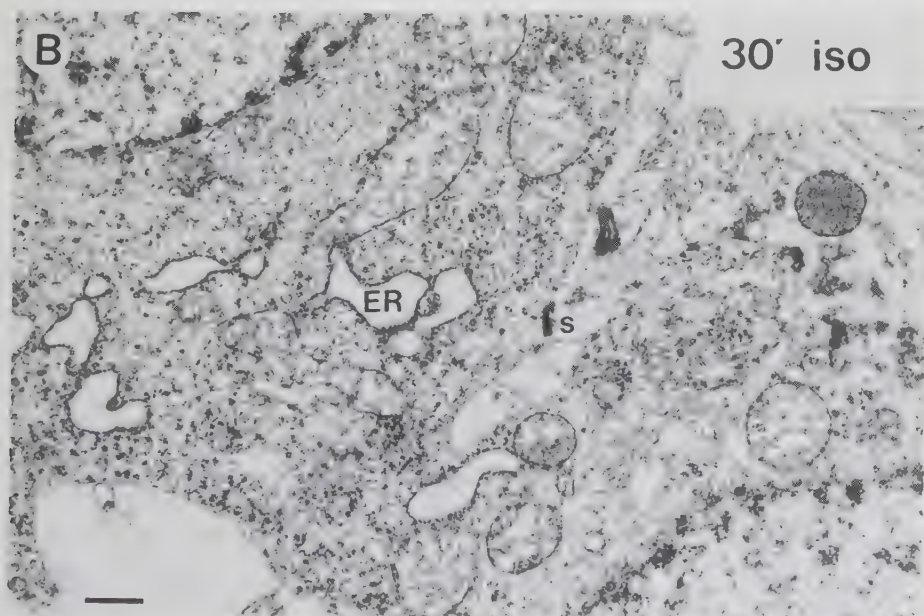
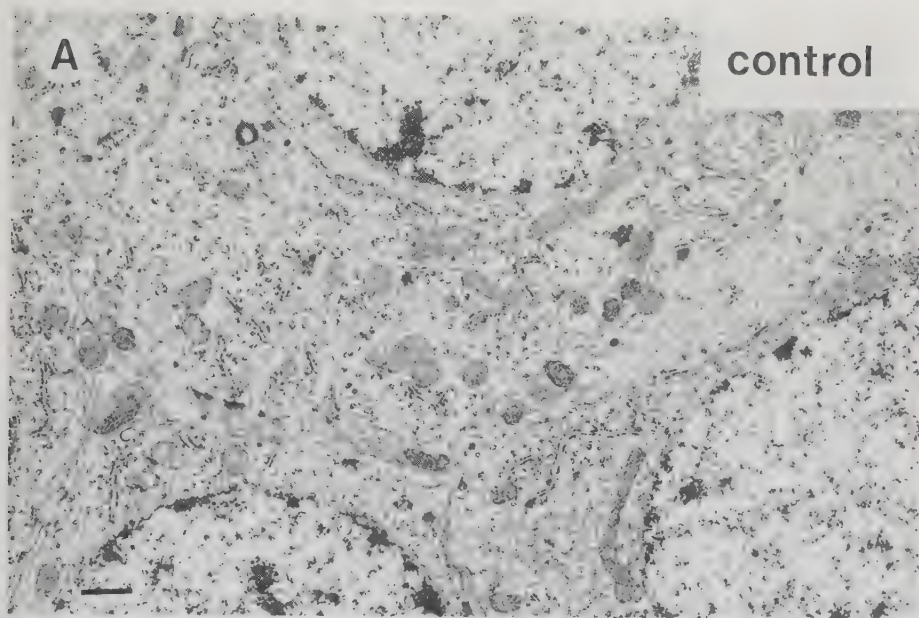
The first pronounced change to occur was in the dendrites of the subpial stratum moleculare, 10 minutes after the onset of cerebral isoelectricity: Severe dendritic swelling was seen, but the

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Figure 6. Dentate granule cell perikarya.

- A. A granule cell body from a rat fixed during delta wave slowing in the EEG shows no abnormalities, although the superficial dendrites (see Fig 4) already show swelling.
- B. After 30 minutes of cerebral isoelectricity, swollen mitochondria with fractured cristae have appeared in the cytoplasm, and the rough endoplasmic reticulum (ER) is dilated. Neurotubules and ribosomes are normal, but cell membrane irregularities have started to appear (lower left). A synapse on the perikaryon is indicated (s). Bar = 1 μ m.

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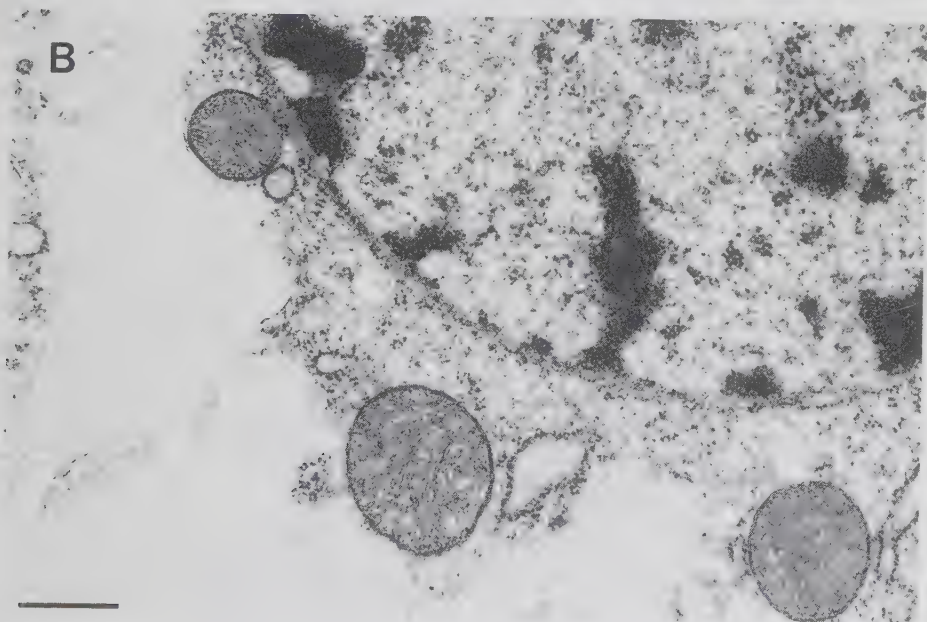
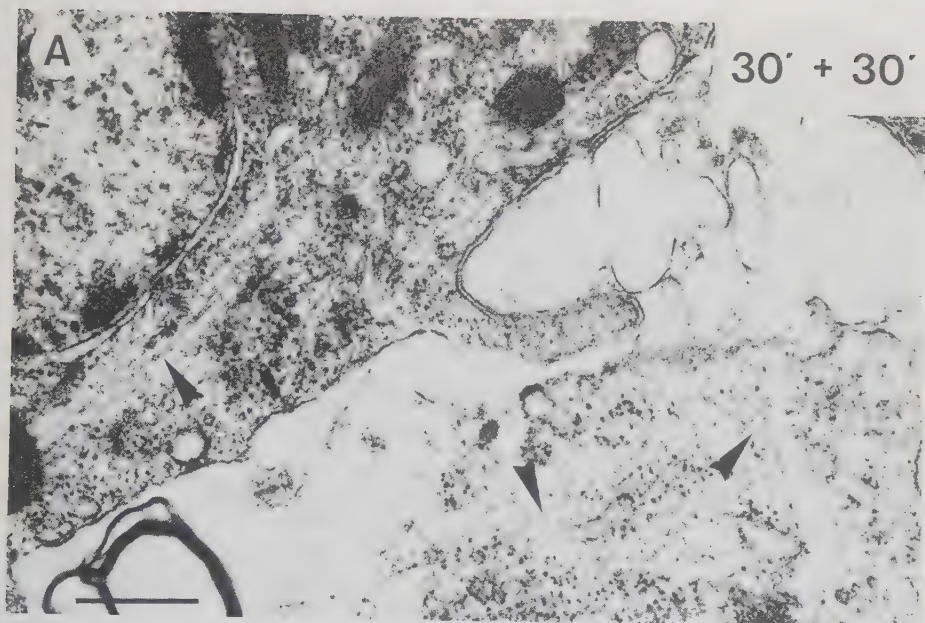


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Figure 7. Neuronal cell membrane damage.

- A. After 30 minutes isoelectricity plus 30 minutes recovery, portions of cell membrane are intact, and portions are ruptured (arrow). Cytoplasmic neurotubules are preserved in both neurons (arrowheads).
- B. Mitochondrial flocculent densities are still absent at this stage, and mitochondrial membranes are still discernible. Bar = 500 nm.

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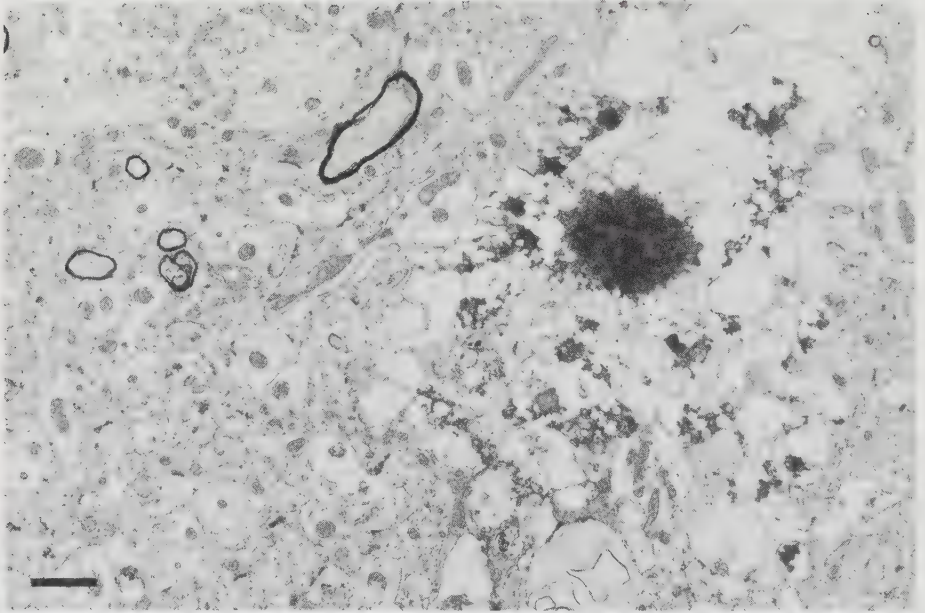


Figure 8. Cytorrhetic neuronal remnant seen in dentate hilus one week after 60 min isoelectricity, a severe hypoglycemic insult. In the axon-sparing nature of the lesion is seen. Relatively intact neuropil, containing some myelinated axons, surrounds a hilus cell with a fragmented appearance corresponding to the latest stages of cell breakdown. Sampling area indicated in figure 2. Bar = 1 μ m.

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intervening axons were spared (Fig. 4B). Synapses were seen on the swollen processes, indicating their dendritic origin (Fig. 5). The dendritic mitochondria were swollen, with fractured cristae, but interspersed axons of passage and terminal axonal boutons contained normal mitochondria (Fig. 5). The neuronal cell bodies were normal at this time, and subsequently up to 30 min of EEG isoelectricity (Fig. 6A). Swollen mitochondria then appeared in the perikaryon, and the rough endoplasmic reticulum became dilated (Fig. 6B). Dark cell transformation was seen in some neurons. Irregularities and minor discontinuities in the cell membranes were seen (Fig. 6B).

After one hour of isoelectricity, and after 30 min isoelectricity followed by 30 min recovery, the appearance was identical. Cell membranes showed focal breaks (Fig. 7A), or were no longer discernible (Fig. 7B). Notably, the cytoskeleton was intact, and mitochondrial flocculent densities were still absent at this time (Fig. 7).

One hour after either 30 or 60 min isoelectricity, cell membranes were absent over large and confluent areas. Mitochondrial flocculent densities appeared, and the cytoplasmic neurotubules and endoplasmic reticulum disappeared. Nuclear chromatin became thick, coarse, and electron dense. These changes have been previously illustrated (6,7,24), and coincided with the appearance of acidophilia by light microscopy.

Neuronal cytolysis ensued in the subsequent week, with concomitant Wallerian degeneration of mossy fiber axons passing to CA3 neurons. However, the neuropil surrounding cytotoxic neurons remained preserved, even in animals with severe damage extending into the dentate hilus, constituting a true, axon-sparing lesion (Fig. 8). The sequence of changes in the dentate neurons is shown schematically in figure 9.

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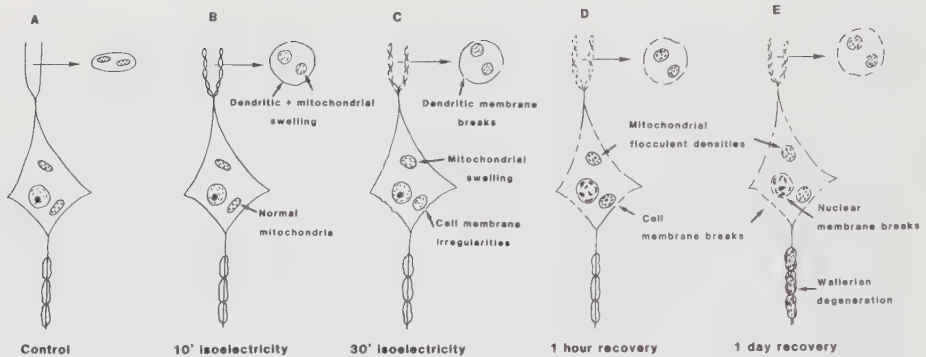


Figure 9. Diagrammatic representation of sequence of cytopathologic events in dentate neurons. Dendrites first become dilated and develop swollen mitochondria. These subsequently appear in the perikaryon. Membrane irregularities, and then breaks also occur first in the dendrites, and later in the perikaryon. Frank membrane rupture, and the development of mitochondrial flocculent densities then occur. Wallerian degeneration and neuronal cytolysis ensue in the following days.

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DISCUSSION

Previous Observations and Selective Vulnerability

Involvement of the dentate gyrus in hypoglycemia was first shown in rabbits by Weil et al in 1938 (47), who observed the blade of the dentate gyrus facing the ventricle to be selectively involved. After due consideration of the vascular (41) and neuronal (45) theories of selective vulnerability they speculated: "But the possibility should also be considered that elimination and distribution of toxic products may take place via not only the vascular system but the cerebrospinal fluid. Therefore one would expect that structures which are closer to the ventricles and subarachnoid spaces are under the influence of higher concentrations of toxic substances dissolved in the cerebrospinal fluid."

Although not discussed, affectation of a large portion of the human dentate gyrus is illustrated in a textbook of neuropathology (9). Others have demonstrated damage to the dentate in man (25) and in rats (4,5,22,51).

A previous article has discussed the relationship of necrotic neurons to white matter and CSF spaces in hypoglycemic brain damage, as well as the literature indicating the flow of cerebral interstitial fluid from gray matter to white matter to the CSF spaces (5). Our present data show necrosis of dentate granule neurons which have dendritic trees exposed to the subarachnoid cisterns. At mid septo-temporal levels of the hippocampus, the dentate crest was characteristically affected first, and with more damage, spreading along the external blade. The most severely affected animals showed necrosis in the internal blade nearer the crest, along the fused hippocampal fissure. It is therefore of interest in this context that radiolabelled CSF enters the

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hippocampal fissure via the Virchow-Robin spaces of the large vessels located in the fissure (26), and that injection of horseradish peroxidase into the subarachnoid space results in a virtually identical distribution of labelling in the rat dentate gyrus (11), to the distribution of dentate neuronal necrosis seen in the present study.

The present findings demonstrate that in explaining selective vulnerability, in addition to the vascular theory of Spielmeyer (41) and the theory of "pathoclisis" of the Vogts (45), consideration must be given to the possibility of toxic extracellular substances in cerebral interstitial fluid and CSF. Although the blood supply (3,10) and neuronal architecture of the rat dentate gyrus and hippocampus have been extensively described (8,12,13,14,17,18,42,43,46,49,52), they reveal no special features of the portions of the dentate undergoing neuronal necrosis. Rather than involving an entire anatomic nucleus or neuronal cell type, damage involves a subset of neuroanatomically homogeneous neurons. Afferent input to the hippocampus may nevertheless play a role in neuronal necrosis due to hypoglycemia.

Spread of damage into the CA4 neurons (2) of the dentate hilus in more severely affected animals, and into CA3 pyramidal neurons is likewise inexplicable on a vascular or purely neuroanatomical basis.

Sequence of Changes in Neuronal Necrosis

The first changes observed in the present study were in the dendritic tree of the dentate granule cells, comprising dilatation of dendrites and swelling of the contained mitochondria. Intermingling axons were spared. By 30 min, swelling of mitochondria and endoplasmic reticulum appeared in the cell body.

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Some degree of mitochondrial swelling in cell bodies and dendrites has previously been shown to be reversible in both hypoglycemia (6) and in ischemia (34), and this stage of cytopathology must therefore be considered potentially reversible.

At one hr following the onset of isoelectricity, an intermediate time between the appearance of reversible and irreversible cellular injury, cell membrane breaks were seen. Although the cell membrane breaks may be artefactual, and absent in vivo, they at least indicate membrane instability to fixation. However, if the former explanation is invoked, it is noteworthy that adjacent mitochondrial membranes were intact (Fig. 7). Cellular repair of the extensive membrane discontinuities seen at one hour seems unlikely. Such cells developed mitochondrial flocculent densities, and regularly underwent necrosis at the dentate crest. However, the possibility that small membrane breaks or discontinuities might be repaired cannot be ruled in or out on the basis of the present findings.

Irreversible cellular injury (6,15,20,23,24,44) in the perikaryon appeared two hrs after the onset of the insult, and was marked by mitochondrial flocculent densities, nuclear and cytoplasmic membrane breaks, and coarse heterochromatic nuclear chromatin. Such irreversibly injured neurons under the electron microscope correspond to the acidophilic neurons of light microscopy seen after a hypoglycemic insult (6) and frequently follow and appear to arise from dark neurons in many brain regions in hypoglycemia (6,7,24).

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Possible Nature of Hypoglycemic Neurotoxin

Of cardinal importance here is not only the relationship of necrotic neurons to the CSF spaces, but also the axon-sparing nature of the lesion in the neuropil. Dendro-perikaryal lesions of the central nervous system, sparing axons of passage and terminal axons have been reported in the nervous system only after administration of excitotoxins, by either intravenous, intracerebral, or intracerebroventricular injection (16,40,49). Early dendritic alterations identical to those seen in the present study, with sparing of intermingling axons, are the characteristic cellular neuropathology of excitotoxic neuronal damage (30,31,32,35,38,39). The limitation of the early changes to dendrites and cell bodies is due to the binding of this class of compounds to neuronal receptors located on the dendritic tree and to a lesser extent on the perikaryon. Axons of passage and axonal boutons terminaux are spared due to a lack of receptors on these portions of the neuron. The progression of the cellular lesion from the neuronal dendrites to the cell body is also accounted for by the dendritic predominance of receptors, and lends support to the hypothesis that the dendritic tree is the vulnerable portion of the neuron, where the cellular injury begins. A similar hypothesis has been proposed in ischemia, based on selective damage to dendritic processes (21).

The chemical identity of an excitotoxin cannot be inferred from the pathological nature of the lesion. Amino acids and their metabolites (36,37), folates (32), cholinergic agents (19,33) are all endogenous compounds of the brain with excitotoxic properties. In hypoglycemia, the excitatory amino acids have been proposed as potential candidates (48). Metabolic alterations during isoelectricity caused by hypoglycemia include changes in the levels

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of excitotoxic amino acids, including a pronounced increase in tissue aspartate (1). A high density of glutamate receptors have been demonstrated in the inner and outer layers of the stratum moleculare, in particular of the N-methyl-d-aspartate type receptor (27,28). Since aspartate indiscriminately kills hippocampal neurons (29) it should be considered as a possible agent accounting for necrosis of the CA3, CA4, and dentate granule neurons seen in the present study. Other agents to be considered, due to their excitotoxic properties, and their derivation from amino acid catabolism, include quinolinic acid (38,39). Definitive identification of the toxin responsible for the neuropathology of hypoglycemic brain damage, must await biochemical investigations.

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